# Identification of the Apoptin tumour specific kinase/s

Cole, Daryl Michael

*Awarding institution:*
King's College London

# Investigation of the Apoptin tumour specific kinase/s

**A thesis presented for the degree of Doctor of Philosophy**

**King's College London**

**Daryl Cole**

**Molecular Oncology**

**Department of Clinical and Diagnostic Sciences**

**King's College London**

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# Abstract

A chicken anaemia virus (CAV) protein, Apoptin, has been shown to induce specific killing targeted at human transformed cells, leaving non-transformed cells unaffected. Investigation has revealed that Apoptin is phosphorylated on a threonine residue in transformed cells, suggesting a role for kinase activation in Apoptin cytotoxicity.

The aim of this study was to identify the kinase or kinases involved in Apoptin cytotoxicity using a serine/threonine kinase siRNA library. In addition, we have recently identified that Apoptin interacts and is phosphorylated by Protein Kinase C (PKC). Also, a potential role for cyclic Guanosine Monophosphate dependent protein kinase (PKG) in the regulation of Apoptin has been investigated. PKC/PKG expression was investigated in cell lines in relation to Apoptin sensitivity. Subsequent modification of PKC/PKG kinase expression was investigated and its effects on Apoptin sensitivity. Further to these studies, a recently discovered human derived gyrovirus producing a protein with homology to CAV Apoptin was investigated.

The results indicated that in HCT116 colon cancer cells Apoptin cytotoxicity was not related to the kinase activity of the siRNA library targets. PKCβ1 was confirmed to be important in sensitivity to Apoptin in multiple myeloma cell lines, however, this importance in Apoptin-induced cell death in head and neck cancer is currently unclear. PKG expression was found to be higher in normal cells compared to transformed cells. In contrast, PKC expression was shown to be higher in transformed cells when compared to normal cells. Apoptin phosphorylation experiments proved to be inconclusive with regards to kinase expression. Physical interaction between Apoptin, PKC and PKG was detected by immunoprecipitation, however, evidence of functional effects based solely on these interactions was not found. The results indicate that PKG and PKC expression is potentially linked to Apoptin function in the cell lines tested. In addition, a newly discovered Human gyrovirus (HGyV) Apoptin was shown to have a similar cellular localisation to CAV Apoptin, and was also found to be toxic to transformed cells, though more indiscriminately than CAV Apoptin.

In conclusion, the results presented here in this study propose a novel role for PKG and PKC in the tumour specific activation of Apoptin in normal and transformed cells. In the absence of PKG, transformed cells activate Apoptin through PKC leading

to nuclear localisation and apoptosis. PKG expression in normal cells appears to block this process, resulting in cytoplasmic localisation of Apoptin and abrogation of Apoptin induced cell death. Additionally, the novel viral protein human gyrovirus Apoptin has been shown to behave in a similar manner to CAV Apoptin, providing a further tumour specific protein for future study. The information obtained during this study will therefore be important in the development of therapeutic strategies for not only determining sensitivity to Apoptin of certain tumours but also for the sensitisation of tumour cells to Apoptin.

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Daryl Cole, 2012

# List of Abbreviations

| | |
|---|---|
| Ad-Apoptin | Adenovirus apoptin |
| Ad-GFP | Adenovirus GFP |
| APC | Adenomatous Polyposis Coli |
| APC1 | Subunit 1 of APC/C |
| APC/C | Anaphase Promoting Complex/Cyclosome |
| Bcl-2 | B-cell lymphoma factor 2 |
| BV-Apoptin | Baculovirus expressing Apoptin |
| CAV | Chicken Anaemia Virus |
| CDKs | Cyclin Dependent Kinases |
| c-FLIP | FLICE-inhibitory protein |
| cGMP | cyclic Guanosine 3', 5' MonoPhosphate |
| DAG | DiAcylGlycerol |
| DAPI | 4', 6-DiAmidino-2-PhenylIndole |
| DD | Death Domain |
| DED | Death Effector Domain |
| DEDAF | DED-Associated Factor |
| DDR | Death Domain Response |
| DISC | Death-Inducing Signalling Complex |
| DMSO | DiMethyl SulfOxide |
| DNA | DeoxyriboNucleic Acid |
| E4orf4 | E4 open reading frame 4 |
| ECL | Enhanced ChemiLuminescence |
| ER | Endoplasmic Reticulum |
| ERKs | Extracellular Signal Regulated Kinases |
| FACS | Fluorescence Activated Cell Sorting |
| FADD | Fas-Associated protein with Death Domain |
| FITC | Fluoroscein IsoThyoCyanate |
| FLIP | FLICE-Inhibitory Protein |
| $G_2$ | Gap 2 |
| GAPDH | GlycerAldehyde 3-Phosphate Dehydrogenase |
| GC-C | receptor Guanylyl-Cyclase |
| GFP | Green Fluorescent Protein |
| GSK-3β | Glycogen Synthase Kinase-3β |

| | |
|---|---|
| H4 | Histone 4 |
| HAMLET | Human Alpha-lactalbumin Made LEthal to Tumour cells |
| HCT116 | Human Colon Carcinoma 116 |
| HGyV | Human GyroVirus |
| Hip-1 | Huntington interacting protein 1 |
| HIV | Human Immunodeficiency Virus |
| HNSCC | Head and Neck Squamous Cell Carcinoma |
| HSC-3 | Head and neck Squamous cell Carcinoma-3 |
| HSC-3M3 | Metastatic Head and neck Squamous cell Carcinoma-3 |
| Hsc70 | Heat Shock Cognate protein 70 |
| HT29 | Human colorectal adenocarcinoma |
| IAP | Inhibitor of Apoptosis Protein |
| J5B | PKG inducible SW620 clone |
| JNK | c-Jun N-terminal Kinase |
| kDa | kilo Dalton |
| Lenti-Apoptin | Lentiviral apoptin |
| Lenti-GFP | Lentiviral GFP |
| LRS | Leucine Rich Sequence |
| M | Mitosis |
| MAPK | Mitogen-Activated Protein Kinase |
| Mda-7 | Melanoma differentiation-associated gene-7 |
| MDM2 | Murine Double-Minute 2 |
| MOI | Multiplicity Of Infection |
| MM1R/S | Multiple Myeloma1 dexamethasone Resistant/Sensitive |
| NCM460 | Normal derived Colon Mucosa |
| NES | Nuclear Export Signal |
| NLS | Nuclear Localisation Signal |
| Nmi | N-Myc interacting protein |
| PARP | Poly (ADP-Ribose) Polymerase |
| PBS | Phosphate Buffered Saline |
| PI | Propidium Iodide |
| PI3K | PhosphatidylInositol 3-Kinase |
| PKB/Akt | Protein Kinase B |
| PKC | Protein Kinase C |
| PKG | cyclic GMP dependent protein kinase |
| PML | ProMyelocytic Leukaemia |
| PMSF | PhenylMethylSulphonyl Fluoride |

| | |
|---|---|
| PTD4 | Protein Transduction Domain 4 |
| PUMA | p53 Upregulated Modulator of Apoptosis |
| Q-RT-PCR | Quantitative Real Time Polymerase Chain Reaction |
| RNA | RiboNucleic Acid |
| SDS | Sodium Dodecyl Sulphate |
| SDS-PAGE | SDS-PolyAcrylamide Gel Electrophoresis |
| siRNA | Short Interfering RiboNucleic Acid |
| SV40 LT antigen | Simian Virus 40 Large T Antigen |
| SW620 | Human Colon Adenocarcinoma |
| TA domain | TransActivation domain |
| TAT | Trans-acting-Activator of Transcription |
| TAT-PTD | TAT Protein Transduction Domain |
| TNF | Tumour Necrosis Factor |
| TRAIL | TNF-Related Apoptosis-Inducing Ligand |
| UMSCC | University of Michigan Squamous Cell Carcinoma |
| VP (1,2,3) | Viral protein (1,2,3) |

# Chapter 1
# Introduction

## *1.1 Cancer*

Cancer is a disease that can be categorised as a genetic disorder caused by mutations either inherited or induced by external factors. Accumulation of mutations leads to changes in cellular signalling and function, changing the characteristics of the cell. In time, these changes can lead to cells that are no longer sensitive to normal homeostatic regulation by the local cellular environment and even by the whole organism (Sarasin, 2003). Many studies have reported that cancer formation is dependent on multiple steps or changes occurring in the cell and surrounding tissues (Doll and Peto, 1978; Peto et al., 2000; Peto et al., 1975). In support of this hypothesis, Hanahan and Weinberg proposed a set of requirements, in 2000 and again in 2011, which seems to apply to the development of all cancers, called the hallmarks of cancer.

Initially, in 2000, the study comprised of 6 individual steps in the development of cancer (Hanahan and Weinberg, 2000), and these included the initiation of angiogenesis, the resistance to cell death, the sustainment of proliferative signalling, the evasion of growth suppressors, the activation of invasion and metastasis and the enabling of replicative immortality. At the time of writing in 2000, these 6 steps were the known developmental factors in cancer formation. By 2011, further advances had seen the addition of two more potential hallmarks of cancer development; (Hanahan and Weinberg, 2011) reprogramming of energy metabolism and the evasion of immune destruction.

The effects these processes have on the cell environment and body as a whole is to disrupt the normal function of tissue homeostasis, which is dependent on the exact balance of cell proliferation, cell death and a combination of positive and negative cellular signals (Vermeulen et al., 2003).

### 1.1.1 Tumour therapy and targets for cancer treatment

Cancer therapy aims to remove cancer cells from the body to prevent further tumour growth. Due to the non-specific nature of current treatment strategies, such as chemotherapy and radiotherapy, unwanted side effects are inevitable. It is because of these side effects that it is very important to develop more specific therapies for cancer treatment that target only tumour cells within the patient.

In order to develop less intrusive methods of treatment with higher efficiency and specificity for cancer, the mechanisms of cancer at the molecular level should be investigated and exploited. For example, the hallmarks of cancer, explained above, describe characteristics applicable to all forms of cancer; in depth study and exploitation of characteristics not present in normal cells could provide potentially tumour specific therapeutic approaches. Many studies have shown that anticancer drugs, in particular, induce apoptosis by varying methods such as activation of caspases, alterations in mitochondrial integrity and function, activation of death inducing ligand or receptor systems and the stabilisation of p53 in sensitive cells (Blagosklonny, 2002; Fulda et al., 1998). All of these processes contribute to similar fates for the tumour cell, for example, changes in chromatin condensation and endonucleolytic cleavage of DNA, indicators of apoptosis. The evidence suggests that different anticancer agents, interacting with different targets in the cell, all depend on a tumour cell's ability to initiate programmed cell death for their cytotoxic function (Hickman, 1992; Hickman et al., 1992).

Changes in the function of certain apoptotic pathways may attribute to the cause of some cancers and could be responsible for increased resistance to cell death (Hanahan and Weinberg, 2011). Increasing the understanding of apoptosis and its function in cancers could therefore lead to methods of sensitising tumours to current therapies and even lead to the production of novel anticancer treatments. In the next section an overview of the current understanding of the apoptosis pathways will be summarised.

## *1.2 Apoptosis*

Apoptosis, or programmed cell death, is a normal mechanism that occurs in healthy tissues during many different processes in the body, including embryonic development, tissue growth and cellular homeostasis. This mechanism is essential in the efficient development of the human body from early growth to ageing and as a tool for maintenance of the correct population size for cells in organs all over the body. In addition to these functions, apoptosis also acts as a defence mechanism against disease or external damaging agents and is also involved in immune reactions removing dead and unwanted cells from the population. Apoptosis can be triggered by a variety of stimuli, both physiological and pathological; however, the same stimulus may not induce the same apoptotic response in different cells (Norbury and Hickson, 2001). As mentioned previously, the acquisition of mechanisms to evade apoptosis is one of the hallmarks of cancer, with both the gain of function of anti-apoptotic and the loss of function of pro-apoptotic signalling contributing to the cancer phenotype and tumourigenesis (Hanahan and Weinberg, 2000). Defective apoptotic mechanisms allow genetically unstable cancer cells to evade destruction and confer resistance to chemotherapy (Cummings et al., 2004; Schmitt, 2003).

Using a combination of light and electron microscopy techniques, the physical changes associated with apoptosis have been described, such as cell shrinkage and chromatin condensation resulting in pyknosis which occur during the early stages (Hacker, 2000; Kerr et al., 1972). Extensive membrane blebbing follows and nuclear fragmentation, known as karyorrhexis, which leads to the separation of cell fragments into apoptotic bodies, a process referred to as budding. These bodies contain cytoplasm, tightly packed organelles and may or may not contain nuclear fragments. The integrity of the organelles and the apoptotic bodies themselves are maintained, thus avoiding damage to surrounding cells and tissues by the release of harmful breakdown products such as cytokines. Ultimately, the apoptotic bodies are phagocytosed by macrophages, parenchymal cells or neoplastic cells and degraded within phagolysosomes (Kurosaka et al., 2003; Savill and Fadok, 2000).

Current research shows that there are two main signalling pathways that lead to apoptosis; the extrinsic pathway, modulated by the death receptor, and the intrinsic pathway, also known as the mitochondrial pathway. DNA damage mainly induces apoptosis via the intrinsic pathway while extracellular signals such as signals from the

cytotoxic cells of the immune system, drive apoptosis through the extrinsic pathway. Induction of either pathway ultimately results in caspase activation, with each caspase potentially activating other procaspases, allowing initiation of a protease cascade (MacFarlane, 2009). Such a cascade, where one caspase activates other caspases, leads to rapid cell death via the amplification of the apoptotic signalling pathway.

There are 11 members of the caspase gene family found in humans and at least 7 of these are important in apoptosis (Riedl and Shi, 2004). Of these 7 apoptotic caspases there are two distinct groups, the initiator caspases and the effector caspases. The initiator caspases comprise of -2, -8, -9 and -10, and the effector caspases include -3, -6 and -7 (Rai et al., 2005).

### 1.2.1 The extrinsic apoptotic pathway

The extrinsic signalling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions through death receptors from the tumour necrosis factor (TNF) receptor family of genes (Locksley et al., 2001). Currently, the most well described ligands and corresponding death receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, TRAIL-R1/DR4 and TRAIL-R2/DR5 (Rubio-Moscardo et al., 2005; Suliman et al., 2001). The defining sequences that occur during the extrinsic pathway are best described by the FasL/FasR and TNF-α/TNFR1 models. Binding of the Fas ligand to the Fas receptor results in binding of the adaptor protein Fas-associated death domain, FADD, and the binding of the TNF ligand to the TNF receptor results in the binding of the adaptor protein TNFR1-associated death domain, TRADD, with the recruitment of FADD and the death domain kinase receptor interacting protein (RIP) (Grimm et al., 1996; Wajant, 2002). FADD subsequently interacts with procaspases-8 via dimerisation of the death effector domain and forms a death inducing signalling complex, called DISC, which results in the activation of caspase-8 (Kischkel et al., 1995). Caspase-8 activation signals the execution phase of apoptosis, however, this can be inhibited by a protein called c-FLIP which bind to caspase-8 and FADD and render them ineffective (Kataoka et al., 1998). The extrinsic apoptotic pathway has also been shown to be linked to the intrinsic pathway via BID cleavage to truncated BID by caspase-8, this leads to outer membrane permeabilisation in mitochondria (Li et al., 1998).

### 1.2.2 The intrinsic apoptotic pathway

The intrinsic apoptotic pathway is mediated by the mitochondria and is triggered by signals that occur intracellularly. The control and regulation of this pathway occurs through the Bcl-2 family of proteins (Cory and Adams, 2002). The Bcl-2 family can be divided into two distinct classes, the pro-apoptotic members and the anti-apoptotic members. The pro-apoptotic Bcl-2 members include Bax, Bak, Bok, Bid, Bad, Bim, Bik, Bmf, Hrk, Noxa and Puma and the anti-apoptotic members include Bcl-2, Bcl-xL, Bcl-w, A1 and Mcl1 (Cory and Adams, 2002). The main mechanism of action of the Bcl-2 proteins is the regulation of cytochrome c release from the mitochondria by regulation of the mitochondrial membrane permeability (Hunter et al., 2007). Cytochrome c binds to and activates Apaf-1 which recruits procaspase-9 forming the apoptosome (Hill et al., 2004). The activation of caspase-9 leads to downstream effector caspase activation resulting in the activation of caspase-3 which induces apoptosis (Hu et al., 1999). Inhibitors of apoptosis (IAPs) inhibit the activation of caspases involved in the intrinsic apoptotic pathway such as caspase-3, -7 and -9 (Roy et al., 1997). In order to counteract the inhibition of apoptosis induced by IAPs, mitochondria can release Smac/DIABLO which directly abrogate the function of IAPs, allowing caspase activation to continue unabated (Ekert et al., 2001).

### 1.2.3 The execution pathway

Both the extrinsic and intrinsic apoptotic pathways culminate in one final pathway considered the final stage of apoptosis, the execution pathway. Execution caspases activate cytoplasmic endonuclease, degrading nuclear proteins, while proteases degrade cytoskeletal proteins. Cleavage of various substrates including cytokeratins, poly (ADP-ribose) polymerase (PARP) and the plasma membrane cytoskeletal protein, α-foldrin, are performed by the executioner caspases (caspase-3, -6 and -7) (Slee et al., 2001). These processes contribute to and result in the biochemical and morphological changes observed during apoptosis. The phagocytic uptake of cells is thought to occur before the final, irreversible DNA fragmentation phase involved in apoptosis. Phospholipid asymmetry and the externalisation of phosphatidylserine on the surface of apoptotic cells and their fragmented bodies is the hallmark of apoptotic phagocytosis. Phosphatidylserine's appearance on the outer surface of apoptotic cells facilitates non-inflammatory phagocytic recognition allowing for early and efficient uptake with minimal inflammatory response (Fadok et al., 2001).

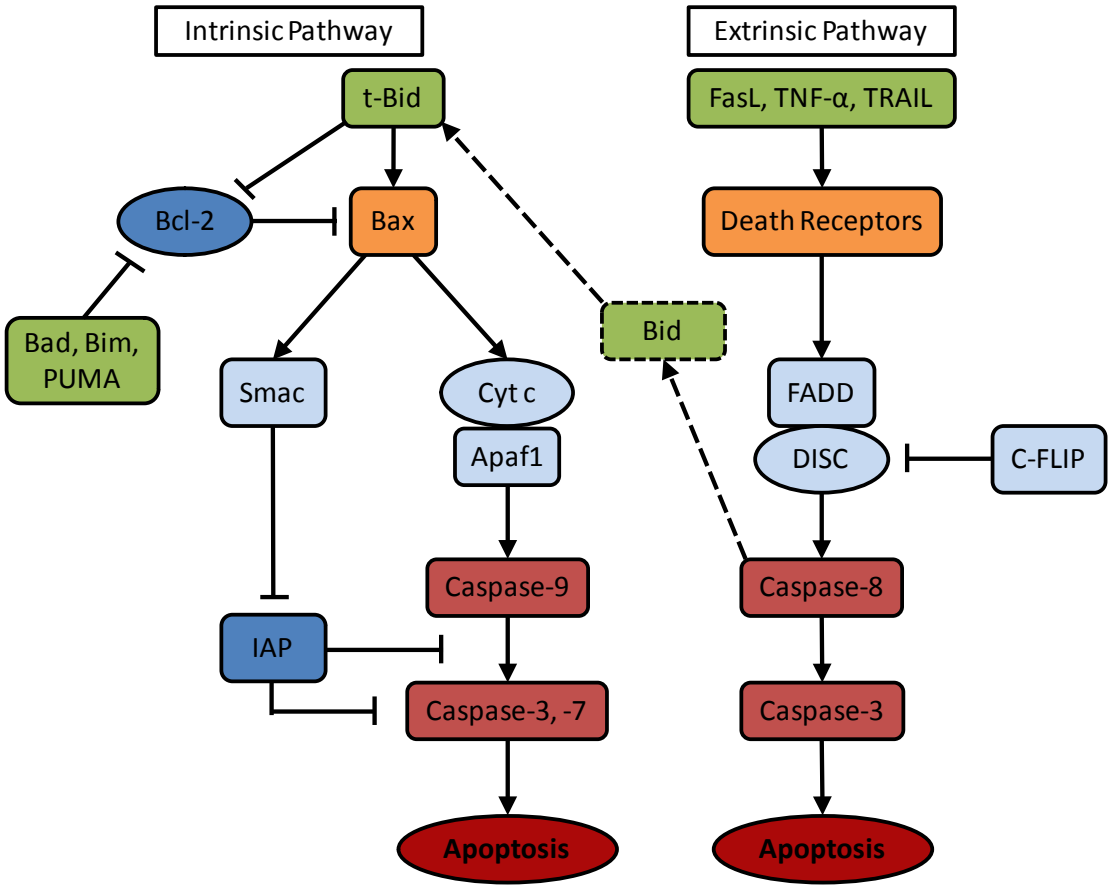



**Figure 1.1 Diagram of the extrinsic and intrinsic apoptotic pathways.** The intrinsic pathway begins with DNA damage and subsequent cytochrome c and Smac release from mitochondria. What follows is the recruitment of Apaf1 by cytochrome c which forms the apoptosome with caspase-9, leading to a cascade of caspase activation and, ultimately, apoptosis. Smac binds to the inhibitor of apoptosis proteins (IAPS) abrogating their inhibition of the effector caspases. The extrinsic pathway begins with the activation of the cell surface death receptors which leads to Fas-associated death domain (FADD) and the death inducing signalling complex (DISC) binding to activate caspase-8 (at this point, caspase-8 can cleave Bid into t-Bid, thus initiating the intrinsic pathway). This induces a cascade of caspase activation leading to apoptosis. This figure was modified from another study (Cory and Adams, 2002).

## *1.3 Tumour specific agents*

As explained in section 1.1.1, tumour specific therapies are very interesting prospects in the treatment of cancers. This section will summarise a number of proteins that specifically kill tumour cells, in particular, originating from cellular or viral sources that have been reported to induce tumour specific apoptosis. These include HAMLET, TRAIL, MDA7, E4orf4 and Apoptin, the focus of this study which will be given a more in-depth review in section 1.3.5.

### 1.3.1 TNF-related apoptosis inducing ligand (TRAIL)

TRAIL is a member of the TNF family of cytokines, with the ability to trigger apoptosis in various tumour cells by activating certain death receptors. To date, five human TRAIL receptors, or death receptors (DR), have been identified including TRAIL-R1 (DR 4), TRAIL-R2 (DR5), the decoy receptors TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), and osteoprotegerin (OPG) (Zhao et al., 2012). TRAIL-R1 and TRAIL-R2 are transmembrane receptors that contain cytoplasmic death domains which act as agonistic receptors of TRAIL. In contrast, TRAIL-R3 and TRAIL-R4 are also transmembrane receptors, but they lack cytoplasmic tails and therefore act as antagonistic receptors of TRAIL. Whether or not OPG is an antagonist for TRAIL is still under investigation due to its low affinity for TRAIL at 37°C (Truneh et al., 2000). Both TRAIL-R1 and TRAIL-R2 are expressed in most human tissues and have minimal cytotoxic effects in normal cells (Chaudhari et al., 2006). TRAIL selectively induces apoptosis in transformed and tumour cells, but does not have this effect in normal cells, making it an interesting agent for cancer specific therapies (Ashkenazi, 2008; Steele et al., 2006; Stieglmaier et al., 2008).

TRAIL induces cell death mainly through the extrinsic pathway, by interacting with TRAIL-R1 and TRAIL-R2 causing conformational changes in these receptors (Scaffidi et al., 1998). This leads to the recruitment of FADD and the formation of DISC, the recruitment of caspase-8 or -10 follows which leads to the activation of caspase-3, -6 and -7 and subsequent cleavage of associated death substrates (Gonzalvez and Ashkenazi, 2010; Kischkel et al., 2000). TRAIL can also induce apoptosis through the intrinsic apoptotic pathway which begins after caspase-8 mediated cleavage of Bid into the proapoptotic truncated Bid (tBid) (Li et al., 1998). This truncated form of Bid

translocates to the outer membrane of the mitochondria where it activates Bax and Bak leading to mitochondrial membrane permeabilisation and subsequent cytochrome c release, which also serves to amplify the extrinsic pathway apoptotic signal (Wu, 2009). In addition, several pro-survival signalling cascades are known to be stimulated by TRAIL such as IKK, Akt and JNK (Abdulghani and El-Deiry, 2010).

### 1.3.2 Human alpha-lactalbumin made lethal to tumour cells (HAMLET)

First described in 1995, HAMLET is a protein discovered serendipitously during the study of the effects of human milk on bacteria (Hakansson et al., 1995). HAMLET is a structural derivative of α-lactalbumin, one of the main proteins found in human milk, and it is this derivative that harbours tumour-selective capabilities (Hallgren et al., 2008).

The process by which HAMLET induces apoptosis in tumour cells is through efficient uptake through direct contact with the plasma membrane, whereby it accumulates in the organelles of the cell; the nuclei, mitochondria, endoplasmic reticulum and proteasomes (Hallgren et al., 2008). The apoptosis inducing function of HAMLET requires the unfolding of α-lactalbumin and the presence of the C18:1 fatty acid as a required cofactor (Mok et al., 2007). In the tumour nuclei, HAMLET binds to histones and disrupts the function of chromatin, particularly affecting the organisation of chromatin within the tumour nuclei leading to loss of transcription and nuclear condensation (Permyakov et al., 2005). *In vitro,* HAMLET has been shown to bind strongly to histones and impair their deposition on DNA (Duringer et al., 2003). In mitochondria, HAMLET causes the release of cytochrome c, through membrane permeabilisation, which results in the activation of the caspase pathways (Kohler et al., 2001). HAMLET can also induce apoptosis in a p53, Bcl-2 and caspase-independent manner in tumour cells (Hallgren et al., 2006). Further to HAMLET's convincing apoptosis capabilities, there is evidence that HAMLET can also induce autophagy in tumour cells (Aits et al., 2009), a lysosomal catabolic pathway that recycles cellular molecules (Scarlatti et al., 2009).

Through clinical trials, HAMLET has successfully proven its selective apoptosis inducing capabilities. Two clinical trials showed that HAMLET was active against skin papillomas and bladder cancer, with no recorded side effects on the adjacent healthy tissues. Of the skin papilloma study, almost all of the papillomas were eradicated from

patients (Gustafsson et al., 2004) and of the bladder cancer study 8 of the 9 treated patients presented significant reductions in bladder tumours (Mossberg et al., 2010). In contrast, however, one study reports that HAMLET can indeed induce apoptosis in normal primary cells, even to a greater extent than some tumour cell lines (Brinkmann et al., 2011). From HAMLET's convincing clinical trials through to its molecular effects in tumour cells, the evidence points to HAMLET as a promising anti-cancer therapeutic with desired tumour specific characteristics. However, as seems to be the case with most tumour specific cytotoxic proteins, the evidence can indicate conflicting functions, suggesting that more study is required to fully understand these proteins.

### 1.3.3 Melanoma differentiation-associated gene- 7 (Mda-7)

Mda-7 (also known as interleukin 24) is a cytokine which has been shown to induce tumour specific caspase dependent apoptosis in a wide range of cells, including melanoma, glioblastoma, osteosarcoma, breast cancer, cervix, lung, colon, nasopharynx, ovary and prostate (Dash et al., 2010; Fisher et al., 2007). Normal cells, for example, melanocytes, astrocytes, fibroblasts, mesothelial and epithelial cells are reported to show minimal effects upon overexpression of Mda-7, suggesting tumour-selective properties (Chada et al., 2004; Dash et al., 2010). In breast cancer cells in particular, Mda-7 has been shown to induce apoptosis by downregulation of Bcl-2 and Akt (Bocangel et al., 2006). Bcl-2 importance in Mda-7 apoptotic function was also shown by overexpression studies that abrogated Mda-7 induced apoptosis upon increased expression of Bcl-2 (Su et al., 2006). Cancer specific apoptosis induced by Mda-7 has also been shown to occur through activation of the FasL/TRAIL pathways (Ekmekcioglu et al., 2008). Clinical trials have shown that Mda-7 is well tolerated and demonstrates significant tumour specific clinical activity (Eager et al., 2008; Emdad et al., 2009; Lebedeva et al., 2007).

### 1.3.4 E4 open reading frame 4 (E4orf4)

The E4orf4 adenovirus derived protein selectively kills tumour cells regardless of p53 status (Lavoie et al., 2000), which suggests that this protein may kill tumour cells that have resisted other conventional therapies (Fuster et al., 2007). A small polypeptide, consisting of 114 residues which shares no extensive sequence homology with any known protein, the product of E4orf4 adenovirus, is reported to induce tumour-

selective apoptosis in its host cell (Kleinberger, 2000). This product is very stable and is shown to persist at high levels even late into infection (Boivin et al., 1999). However, for normal viral replication, the E4orf4 product is not essential and an exact role for this product in the lytic infection of the E4orf4 adenovirus is not clearly understood (Lavoie et al., 2010).

The apoptotic pathways activated by E4orf4 are diverse and include p53-independent (Shtrichman and Kleinberger, 1998) and caspase independent cell death (Lavoie et al., 1998). Recently, it has also been shown that mitochondrial release of cytochrome c is not required for E4orf4 induced apoptosis (Li et al., 2009). Phosphorylation of the E4orf4 product is modulated by the Src-family kinases and it has been shown that the E4orf4 product interacts with protein phosphatase 2A (PP2A) (Lavoie et al., 2010). Studies suggest that the interaction of the E4orf4 protein product with the Src-family kinases and PP2A result in its apoptotic action (Marcellus et al., 2000; Shtrichman et al., 1999). E4orf4 appears to activate apoptosis through alternative death pathways as it does not require classical caspase pathways and circumvents Bcl-2 blockage of cell death (Champagne et al., 2004).

### 1.3.5 Apoptin

Apoptin was discovered during the study of the first known gyrovirus, chicken anaemia virus (CAV), a virus that has been reported to induce apoptosis in chicken thymocytes and lymphoblastoid cells (Noteborn et al., 1994), hence the name chicken anaemia virus. Three proteins are encoded by CAV; VP1 (a capsid protein), VP2 (a scaffold protein and protein phosphatase) and VP3 (Backendorf et al., 2008b). VP3 is the most interesting protein produced by CAV as it possesses the same apoptotic ability as the virus (Noteborn et al., 1991; Zhuang et al., 1995b) and because of this it was renamed Apoptin (Backendorf et al., 2008b).

At 121 amino acids in length, Apoptin is a protein rich in prolines, serines, threonines and basic amino acids (Noteborn et al., 1991). In addition to its apoptotic ability in chicken thymocytes and lymphoblastoid cells, Apoptin has been shown to have tumour specific cytotoxicity in human tissues and cells, for example primary fibroblasts, keratinocytes, smooth muscle cells, T-cells and endothelial cells are not killed by Apoptin (Backendorf et al., 2008b; Danen-Van Oorschot et al., 1997; Guelen et al., 2004; Zhang et al., 2003; Zhuang et al., 1995b). Although Apoptin has been

shown to not induce apoptosis in a variety of normal cells, the transformation of such cells using SV40 Large T antigen does create cells that are sensitive to Apoptin induced cell death (Danen-Van Oorschot et al., 1997).

Recent studies have revealed the discovery of a new gyrovirus found in humans called human Gyrovirus (HGyV) and, interestingly, this virus also encodes a protein with sequence homology to CAV Apoptin (Sauvage et al., 2011). This protein will be reviewed in further detail in section 1.3.5.6.

### 1.3.5.1 The tumour specific characteristics of Apoptin

Further understanding of Apoptin's tumour specific function is required to potentially exploit its characteristics in a clinical setting, and in order to do this, the structural and molecular properties of the protein were initially investigated. Studies revealed that Apoptin contains a bipartite nuclear localisation signal formed of nuclear localisation signal 1 and nuclear localisation signal 2 (NLS1 and NLS2) at amino acids 82-88 and 111-121(Figure 1.2), respectively (Danen-Van Oorschot et al., 2003). In addition, Apoptin contains a hydrophobic sequence that resembles a nuclear export signal (NES) (Figure 1.2), which suggests that the protein has the ability to shuttle between the nucleus and the cytoplasm of cells (Noteborn et al., 1991). The studies showed that both NLS1 and NLS2 could function independently of each other, however, both regions were required for efficient nuclear localisation (Danen-Van Oorschot et al., 2003). In addition, Apoptin possesses a Leucine Rich Sequence (LRS) at its N-terminal end (Figure 1.2) important for the protein's binding ability not only to other proteins such as promyelocytic leukaemia (PML) protein, but to other Apoptin molecules facilitating its multimerisation and aggregation (Argiris et al., 2011). This sequence was initially thought to be a second NES; however a more recent study has shown that this LRS actually contributes to nuclear accumulation of Apoptin such that in its absence a marked reduction in tumour specific Apoptin nuclear localisation is observed (Oro and Jans, 2004; Poon et al., 2005; Wadia et al., 2004).

A reported characteristic of Apoptin's behaviour in cells is its ability to form complexes with other proteins and subsequently interact with DNA via the LRS (Leliveld et al., 2003). For example, in tumour or transformed cells, Apoptin localises to the nucleus where it colocalises mostly with heterochromatin and nucleoli and induces apoptosis (Leliveld et al., 2003; Zhang et al., 2003). Apoptin is also observed to localise

to PML bodies in the nucleus of tumour cells and this behaviour is reported to be dependent on the LRS (Poon et al., 2005). In normal cells, however, Apoptin does not localise to the nucleus and remains in the cytoplasm, where it is physically unstable. It then disperses into fine particles and accumulates slowly into larger aggregates, at which point it moves to the plasma membrane and no traces remain in the cell (Zhang et al., 2003). The difference in apoptotic function in tumour and normal cells correlates with the observed changes in cellular localisation, because of this it was hypothesised that nuclear localisation was essential for Apoptin induced apoptosis. Upon forced nuclear expression of Apoptin in normal cells, however, it was shown that cell death was not induced, this suggested that Apoptin induced apoptosis required more than just nuclear localisation for its function (Danen-Van Oorschot et al., 2003).

Studies on Apoptin also revealed another interesting characteristic that would benefit future understanding of the protein. Phosphorylation occurs on Apoptin in tumour cells and not in normal cells on a specific residue, Threonine 108 (T108), however, the kinase responsible is currently unknown (Rohn et al., 2002). Further supporting this, a gain of function point mutation study of the Apoptin T108 residue by substituting it for glutamic acid, found that Apoptin was able to enter the nucleus of normal cells and induce apoptosis (Backendorf et al., 2008b; Rohn et al., 2002). Thus suggesting that phosphorylation of T108 is very important in the nuclear localisation of Apoptin and the activation of Apoptin induced apoptosis. The variation and number of tumour and transformed cells that are killed by Apoptin suggests that these cells perhaps share similar characteristics that are exploited by Apoptin to induce apoptosis and that this cell death may follow the same route of activation in these cells. Because of this, and the potential importance of phosphorylation for Apoptin's function, it is important to further understand and identify a potential tumour specific kinase involved in Apoptin induced apoptosis. Such a finding could lead to the development of new cancer therapeutics that exploit Apoptin's exciting properties.

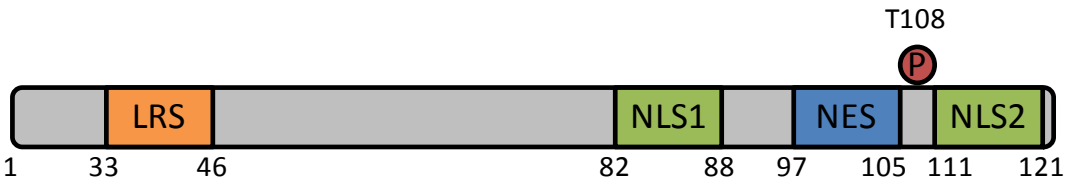



**Figure 1.2 Diagram of Apoptin's structure.** The nuclear localisation signals 1 and 2 (NLS1 and NLS2) are highlighted in green, the nuclear export signal (NES) is highlighted in blue and the leucine rich sequence (LRS) is highlighted in orange. The Threonine 108 (T108) phosphorylation site is indicated by the red circle. This figure was modified from another study (Los et al., 2009).

### 1.3.5.2 Mechanisms of Apoptin mediated cell death

Apoptin mediated apoptosis appears to involve the mitochondrial death pathway and has been shown to coincide with the release of cytochrome c, a key step in the intrinsic apoptotic pathway (Danen-van Oorschot et al., 2000). Activation of caspase-3, a key downstream effector caspase, is also observed during Apoptin mediated cell death. In addition, a deficiency in Apaf1, a target of cytochrome c, strongly protects tumour cells from Apoptin induced apoptosis (Burek et al., 2006). It has also been shown that Apoptin induced cell death does not occur through the extrinsic pathway. For example, cells deficient in FADD or caspase-8, upstream targets of the extrinsic pathway, were shown to be just as sensitive to Apoptin mediated cell death as their parental clones (Maddika et al., 2005). Further work showed that both caspase-8 and also caspase-1 were not involved in Apoptin cell death as inhibitors of these cysteine proteases did not affect cells sensitivity to Apoptin cytotoxicity (Danen-van Oorschot et al., 2000).

Intriguingly, the role of anti-apoptotic Bcl-2 in Apoptin's function is still not completely clear, studies have shown some contrasting findings. In one study, Apoptin function was found to be independent of Bcl-2 in human osteosarcoma Saos-2 and U2OS cells (Zhuang et al., 1995a). Another study found that the Bcl-2 protein accelerated Apoptin induced cell death (Danen-Van Oorschot et al., 1999). In contrast to the above findings, one study showed that Apoptin induced cell death was inhibited by the overexpression of both Bcl-2 and Bcl-xL in human breast cancer MCF-7 and human medullablastoma D283 cells (Burek et al., 2006). These contrasts in the study of Apoptin cytotoxicity relative to Bcl-2 activity may be explained by inherent differences in specific tumour cells and, more precisely, the cellular factors that control and regulate Bcl-2 and its anti-apoptotic function.

Another Bcl-2 protein, BAX (a pro-apoptotic member of the Bcl-2 family), was shown to sensitise cells to Apoptin induced apoptosis, cells found to lack BAX expression were strongly protected (Burek et al., 2006). Together, the data described here suggests that Apoptin induced cell death is mediated by the Bcl-2 family, involves the activation of caspases and mitochondrial release of cytochrome c via the intrinsic apoptotic pathway. Nur77, a nuclear orphan receptor and a member of the steroid receptor family, perhaps sheds some light on the controversies surrounding Bcl-2 and Apoptin mediated cell death. It has been reported that Nur77 can change the function

of Bcl-2 from an anti-apoptotic to a pro-apoptotic molecule leading to the activation of the mitochondrial death pathway (Lin et al., 2004a). The varying levels of Nur77 found in different cell types could, therefore, explain the differing effects of Bcl-2 on Apoptin induced cell death. In addition, translocation of Nur77 from the nucleus to the cytoplasm in MCF7 cells has been shown to be triggered by Apoptin and siRNA knockdown of Nur77 protected MCF7 cells from Apoptin mediated apoptosis (Maddika et al., 2005).

#### 1.3.5.3 Molecules that interact with Apoptin

Several cellular proteins have been described that interact with or bind to Apoptin and affect its cellular localisation and its subsequent apoptosis inducing ability. Peptidyl-prolyl isomerase-like 3 (Ppil3), which belongs to the cyclophilin family, has been reported to interact with Apoptin. The members of this family play a role in mitochondrial maintenance, cell cycle control and apoptosis, processes linked to Apoptin induced cell death. Knockdown of Ppil3 by siRNA was shown to lead to the translocation of Apoptin from the cytoplasm into the nucleus, while expression of the protein caused Apoptin to migrate to the cytoplasm in tumour cells (Huo et al., 2008). Another reported binding partner for Apoptin is Hippi, a protein that interacts with and is an apoptosis co-mediator of huntington-interacting protein 1 (Hip 1). In normal cells, Apoptin and Hippi colocalise in the cytoplasm, but in tumour cells, Apoptin localises to the nucleus leaving Hippi in the cytoplasm (Cheng et al., 2003).This suggests that Ppil3 and Hippi may play a role in the cytoplasmic localisation of Apoptin in normal cells.

Other proteins that have been shown to interact with Apoptin include Nmi (N-Myc interaction protein, which binds to DNA) (Backendorf et al., 2008b) and E1B-21kDa (the small adenovirus 5 E1B protein of 21kDa which inhibits Apoptin induced cell death) (Zhuang et al., 1995a). More recently, Gallus heat shock cognate protein 70 (Hsc70) was reported to associate with Apoptin (Chen et al., 2011b). Knockdown of Hsc70 resulted in inhibition of Akt phosphorylation by Apoptin, suggesting that Hsc70 may be essential in the phosphorylation of Akt induced by Apoptin (Chen et al., 2011a).

APC1, a subunit of the anaphase-promoting complex/cyclosome (APC/C), has been shown to associate with Apoptin. This interaction results in the inhibition of APC/C ubiquitin ligase function causing $G_2$/M arrest and apoptosis in p53-null transformed cells (Teodoro et al., 2004b). This interaction is present in tumour cells but

not in normal cells, suggesting another possible tumour selective mechanism of Apoptin function. Apoptin expression in transformed cells can also initiate the formation of promyelocytic leukaemia (PML) bodies and the recruitment of APC/C to these nuclear structures (Heilman et al., 2006; Janssen et al., 2007).

Apoptin can also interact with a pro-apoptotic protein called death effector domain-associated factor (DEDAF). This protein has been found to associate with death effector domain (DED)-containing pro-apoptotic proteins and colocalises with Apoptin in the nucleus of cancer and transformed cells, but not in primary fibroblasts or mesenchymal cells (Danen-van Oorschot et al., 2004). Co-expression of DEDAF and Apoptin was shown to increase the rate of apoptosis when compared to either protein expressed alone (Danen-van Oorschot et al., 2004). It has also been reported that Apoptin colocalises with FADD and Bcl-10, a caspase recruitment domain-containing protein, in cytoplasmic structures called death effector filaments (Guelen et al., 2004).

#### 1.3.5.4 Phosphorylation of Apoptin and the importance of threonine 108

As described previously, the process by which Apoptin selectively targets tumour cells for destruction is currently unknown. Phosphorylation on Threonine 108 (T108), however, appears to play a role in the cellular localisation of Apoptin, but the kinase responsible has yet to be identified (Rohn et al., 2002). Mapping the structure and sequence of Apoptin revealed that T108 is adjacent to the nuclear export signal (NES) implying that phosphorylation of T108 seems to induce tumour specific localisation of Apoptin through the inactivation of the NES (Rohn et al., 2002). However, one study reported that T108 phosphorylation does not play as big a role in Apoptin's pro-apoptotic function as was previously thought (Lee et al., 2007). However, there is a cluster of Threonine residues (aa 106-108) within the Apoptin sequence that could still facilitate the phosphorylation of Apoptin in the presence of T108 mutation. In support of this theory, one study showed that mutation of T108 was not sufficient to abrogate Apoptin's apoptotic function; both T108 and T107 were required to be mutated to completely abolish Apoptin induced cell death (Rohn et al., 2005).

The importance of Apoptin phosphorylation in its tumour specific function has made the search for an Apoptin kinase a priority in understanding the protein's activity in tumour cells. Previous work had identified the cyclin-dependent kinase, CDK2, as a potential Apoptin kinase required for Apoptin induced apoptosis. In PC3 and MCF7 cell

lines, Apoptin was shown to interact with phosphatidylinositol 3-kinase (PI3-kinase) which leads to activation and nuclear localisation of Akt (Maddika et al., 2008). In the nucleus, Akt then activates CDK2 by direct phosphorylation and, in addition, phosphorylation induced degradation of $p27^{Kip1}$ by the proteasome-dependent pathway (Maddika et al., 2008). Dominant-negative mutants of PI3-kinase and Akt, as well as inhibitors of these molecules, reduced CDK2 activation and also inhibited Apoptin induced apoptosis. Additionally, CDK2-deficient fibroblasts and CDK2 knockdown cells displayed reduced sensitivity to Apoptin induced cell death (Maddika et al., 2009).

Recent work by our group has identified an isoform of the protein kinase C (PKC) family of kinases as a potential Apoptin kinase. The study suggests that PKCβ regulates the translocalisation of Apoptin into the nucleus and induces apoptosis in human multiple myeloma (MM) cell lines (Jiang et al., 2010a). Affymetrix microarray analysis revealed that PKCβ was a potential Apoptin kinase candidate. In support of this, *in vitro* immunoprecipitation studies found that PKCβ was responsible for Apoptin phosphorylation (Jiang et al., 2010a). Subsequent PKCβ knockdown, using shRNA techniques, showed a significant decrease in Apoptin phosphorylation. Apoptin induced apoptosis was shown to occur through the upregulation of PKCβ, the activation of caspase-9 and caspase-3, cleavage of the PKCδ catalytic domain and downregulation of the proto-oncogene tyrosine-protein kinase MER (MERTK) (Jiang et al., 2010a). In contrast to previous Apoptin kinase work, this study also demonstrated that Apoptin induced apoptosis was activated through the downregulation of Akt.

Additionally, recent studies have found that Apoptin localisation is dependent on DNA damage response (DDR) signalling, for example, induction of DDR signalling in normal primary cells resulted in nuclear localisation and apoptosis. In contrast, inhibition of DDR signalling through RNA interference of DDR factors such as DNA-dependent protein kinase (DNA-PK) resulted in cytoplasmic localisation of Apoptin in transformed cells. In addition, Apoptin was observed to inhibit the formation of DNA damage foci in normal cells, suggesting a novel mechanism of DDR inhibition during viral infection (Kucharski et al., 2011).

#### 1.3.5.5 The clinical potential of Apoptin

Apoptin's ability to induce apoptosis specifically in tumour cells has been shown in several studies and, in addition to this, a number of *in vivo* studies have been

published that provide intriguing insights into Apoptin's potential in the clinical environment. Several preclinical studies have also shown that Apoptin can improve the efficacy of existing therapies, highlighting another potential therapeutic role for Apoptin.

*In vivo* studies using HepG2 cells xenografted into nude mice demonstrated that only a single injection of adenoviral Apoptin intratumourally resulted in a significant reduction in tumour mass. This observation also coincided with no serious side effects when the Apoptin virus was delivered intravenously, subcutaneously or via intraperitoneal injection in healthy rats (Pietersen et al., 1999). A further xenograft study in immune-deficient mice using human hepatoma cells revealed that multiple injections of adenoviral Apoptin decreased tumour mass in addition to prolonging survival in 60% of mice treated (van der Eb et al., 2002). Interestingly, after 6 months, 30% of the treated mice remained tumour free.

Another Apoptin expressing virus that has been used in *in vivo* studies is baculovirus which was shown to induce apoptosis in H22 murine hepatoma cells grafted into C57BL/6 mice. The reported effects of intratumoural delivery of this virus were significant suppression of tumour growth and a significant increase in length of survival of the mice carrying tumours in comparison to those treated with a control virus expressing EGFP (Pan et al., 2010).

Further *in vivo* studies in mice used a cancer specific promoter, called human telomerase reverse transcriptase promoter (hTERT). This promoter was combined with Apoptin in an adenoviral vector to form Ad-hTERT-E1a-Apoptin to produce E1a-Apoptin that was driven by the hTERT promoter. Intratumoural injection of this adenovirus in C57BL/6 mice with B16 xenografted cells resulted in the reduction of tumour mass and an increase in animal survival (Li et al., 2010). A similar study also showed similar results in gastric carcinoma in nude mice that had established primary tumours; however, improvements in tumour burden and mouse survival were seen in both intratumoural injection and systemic delivery of Ad-hTERT-E1a-Apoptin (Liu et al., 2012).

As previously mentioned, Apoptin has shown potential in combination therapy with other more conventional anti-cancer treatments. For example, radiotherapy has been shown to have enhanced efficacy upon combination treatment with Apoptin. Using radioresistant SQD9 human head and neck squamous carcinoma cells, concurrent treatment with irradiation and exposure to Apoptin produced cytochrome c

release and cleavage of caspase-3. Irradiation alone, however, did not induce the same apoptosis associated events in SQD9 cells (Schoop et al., 2010). Further to this, delivery of the TRAIL and VP3 genes (pBud-TRAIL-VP3) to gastric cancer cells by attenuated Salmonella typhimurium was shown to significantly diminish the size and growth of SGC-7901 xenografts in nude mice (Cao et al., 2010).

A combination of delivery mechanisms for Apoptin has also been tested *in vivo* using a lentiviral construct to deliver secretable TAT-Apoptin. The addition of a secretory signal peptide was made to transacting activator of transcription (TAT)-Apoptin to produce a virus capable of secreting Apoptin from its infected hosts and using the TAT delivery system to deliver Apoptin to neighbouring cells. Results using TAT-GFP as a control showed that the TAT-Apoptin virus, delivered systemically, eradicated the hepatocellular carcinoma xenograft tumours in the nude mice, while the xenografts that received the TAT-GFP control continued to grow (Ma et al., 2012). This treatment was also shown to have a low cytotoxicity in the animals used.

Other approaches to Apoptin delivery have also been studied and show great promise. One of these methods of delivery, mentioned briefly above, is the TAT or transacting activator of transcription. This method of protein transduction is most commonly used and comprises of a positively charged domain found in the HIV-1 trans-activator protein TAT called the TAT protein transduction domain (TAT-PTD) (Ho et al., 2001). TAT-Apoptin has been shown to be efficiently transduced into both normal and tumour cell lines, where the normal cells (1BR3 and 6659 fibroblasts) were not killed, but the tumour cells (Saos-2 and HSC-3) were sensitive and became apoptotic (Guelen et al., 2004). Protein transduction domain 4 (PTD4), a domain similar to TAT with minor modifications, has also been shown to efficiently deliver proteins across plasma membranes and significantly inhibit tumour growth *in vivo* when tagged to Apoptin (PTD4-Apoptin) (Sun et al., 2009). Using combination treatment with PTD4-Apoptin and dacarbazine in C57BL/6 mice bearing subcutaneous B16-F1 melanoma tumours produced a synergistic anti-tumour effect (Jin et al., 2011).

Recently, TAT has been incorporated into the wheat histone H4 in combination with luteinising hormone releasing hormone (LHRH) to produce H4TL, or H4-TAT-LHRH. The aim of this genetic manipulation was to create an enhanced form of H4 that more efficiently crossed the plasma membrane to deliver Apoptin to LHRH receptor-positive cells which, in the case of this study was the ovarian cancer HO8910 cells (Wang and Zhang, 2011). Transfection of these cells with H4-TAT-LHRH resulted in an

increase in Apoptin expression at both the mRNA and protein level which coincided with reduced growth of HO8910 cells and loss of mitochondrial membrane potential (Wang and Zhang, 2011).

In summary, the data reported in the studies described here suggest a very clear potential for Apoptin as a tumour specific anti-cancer therapeutic. In addition, Apoptin possesses some unique characteristics that, if developed further, could make it an important therapeutic in situations where conventional therapies have failed. Moreover, observations presented here indicate that Apoptin, as a combination therapy with conventional chemotherapeutics, may offer an effective strategy for cancer treatment.

#### 1.3.5.6 Human gyrovirus (HGyV) and its Apoptin homologous protein

As mentioned previously, when CAV was discovered it was the first known gyrovirus, however, recent work has identified a virus originating in humans from this family, a virus called human gyrovirus (HGyV). The interesting characteristic of this virus is that it produces a protein with sequence homology to VP3/Apoptin that is produced by CAV (Sauvage et al., 2011).

Work produced by our lab further investigated the potential of this protein in human cancers. Results indicated that HGyV-Apoptin behaved in a very similar manner in both normal and tumour cells as CAV-Apoptin. For example, in normal 1BR3 fibroblasts, HGyV-Apoptin was found to localise in the cytoplasm and in Saos-2 tumour cells and 1BR3 SV40 Large T transformed (1BR3LT) cells, HGyV-Apoptin localised to the nucleus, where induction of apoptosis was evident (Bullenkamp et al., 2012). These results were shown to compare favourably with CAV-Apoptin, which produced similar results, suggesting that HGyV-Apoptin may possess anti-cancer properties in line with CAV-Apoptin's well documented tumour specific characteristics.

## *1.4 Kinases*

Many, if not all cellular processes require enzymatic catalysis of one kind or another, for example, the cleavage of one molecule into another form, the modification of a molecule that facilitates its function, or the complete breakdown of a molecule into its constituent parts. Each of these processes requires some form of enzyme activity and the most important aspect of enzyme activity is specificity. The importance of this specificity is that it ensures that processes that occur in the body are performed correctly and are well regulated.

Proteases are a form of enzyme and their role is to facilitate proteolysis, the hydrolysation of protein peptide bonds, which also occurs during apoptosis but, in addition is involved in the digestion of food and the blood-clotting cascade. Caspases are a subdivision of proteases, called cysteine proteases that catalyse the cleavage of proteins into new products usually activating them in the process, leading to the caspase cascade which is seen during apoptosis.

Another group of enzymes, known as the transferases, is responsible for catalysing the transfer of functional groups from one molecule to another. In biological systems, the two main groups of transferases are polymerases and kinases. Polymerases are facilitators of DNA and RNA polymerisation by transcription and replication of new DNA or RNA from an existing template using nucleotides to catalyse synthesis of polynucleotide strands.

Kinases are a group of enzymes that are involved in many regulatory biological processes, relaying signals from outside the cell to cause downstream events, such as cell metabolism, migration or proliferation. Kinases comprise 1.8% of the human proteome, using ATP to catalyse the transfer of γ-phosphate of ATP to serine, threonine or tyrosine residues (Manning et al., 2002). Modification of protein residues by kinase interaction results in conformational, structural and functional changes, activating or inactivating the target substrate (Figure 1.3). This can then lead to the activation of signalling pathways and a cascade of kinase activity and protein phosphorylation resulting in a variety of cellular functions, from proliferation and cell survival, to cell cycle arrest and apoptosis.

Unregulated activity of certain kinases is the cause of many cancers, and this link has been proven by the successful use of kinase inhibitors in the treatment of cancer (Boyle and Koleske, 2007).

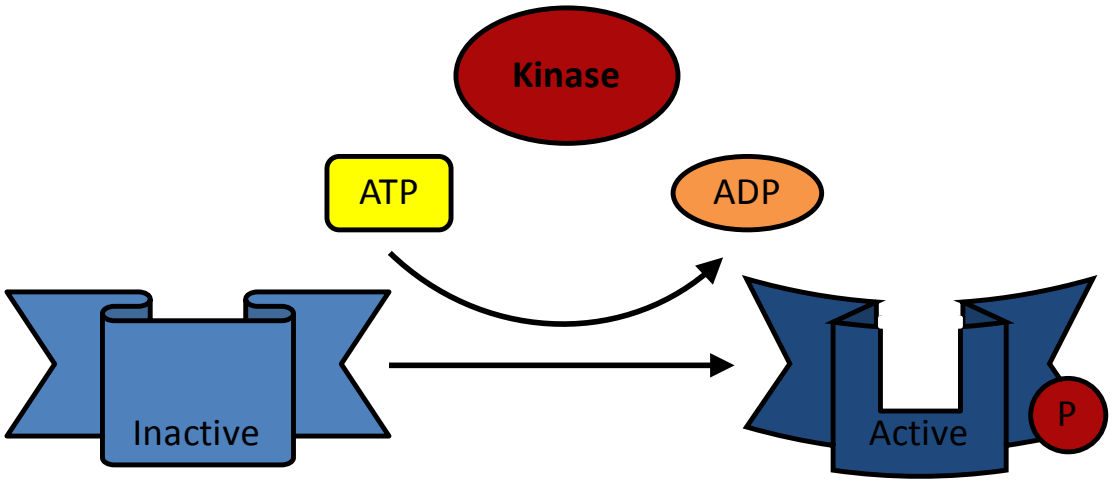



**Figure 1.3 Diagram describing the mechanism of kinase mediated phosphorylation of a substrate.** A kinase interacts with its substrate by catalysing the transfer of a phosphate group from ATP to the side chains of the substrates residues. This results in a conformational change and subsequent functional changes from an inactive form into an active form of the substrate. This figure was modified from another study (Kosior et al., 2011).

### 1.4.1 Kinase inhibitors and their involvement in cancer therapy

As mentioned previously, a large number of studies on the subject of kinase activity and cancer have been published and kinase inhibitors, for example, are already commercially available as anti-cancer therapy options.

Imatinib is a tyrosine kinase inhibitor which was found to selectively inhibit the platelet-derived growth factor (PDGF) receptor, c-kit (Tuveson et al., 2001), BCR-Abl, c-Abl and Abl-related gene (Arg) at concentrations below the μM level (Buchdunger et al., 1996). Imatinib targets cancer by inhibiting the tyrosine kinase activity of these kinases which are upregulated in certain cancers. Imatinib is used primarily to treat chronic myelogenous leukaemia (CML) because the cause of over 95% of these tumours is fusion of the BCR and Abl genes resulting in a constitutively active kinase known as BCR-Abl (Advani and Pendergast, 2002; Cortez et al., 1995). The specific targeting and inhibition of BCR-Abl by Imatinib results in a 95% haematologic response with minor side effects (O'Dwyer et al., 2003).

Another kinase inhibitor, lapatinib, is used in the treatment of breast cancer and targets the kinase domain of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2). In studies, Lapatinib was shown to block the downstream signalling of the Akt and mitogen-activated protein kinase (MAPK) in human tumour xenografts (using HN5 cells in nude mice) (Xia et al., 2002) and SKBR3 breast cancer cells (Nahta et al., 2007). Other EGFR inhibitors include cetuximab (a monoclonal antibody that blocks the EGFR receptor), which is used to treat metastatic colorectal cancer and head and neck cancer, gefitinib and erlotinib, small molecule inhibitors that are used to treat non-small-cell lung cancer (Morphy, 2010).

SRC kinase is involved in multiple processes relevant to both prostate and breast cancers including bone activity, tumour growth, hormone receptor signalling/resistance and metastasis (Saad, 2009). Inhibition of SRC kinase activity with dasatinib in mice resulted in reduced prostate tumour growth in bone (Yano et al., 2008) and significantly prevented the formation of osteolytic metastases (Zhang et al., 2009). In human trials, dasatinib was shown to reduce the levels of urinary N-telopeptide, a marker of adverse outcomes in men with prostate cancer bone metastases, in 80% of patients treated (Yu et al., 2009).

The mammalian target of rapamycin (mTOR) kinase is a key element of intracellular signal transduction, responsible for the regulation of cell growth and proliferation (Grzybowska-Izydorczyk and Smolewski, 2012). Rapamycin was first described as an anti-fungal agent but later became known for its immunosuppressant properties. Subsequent studies found that rapamycin had an anti-cancer activity against a large variety of cell lines derived from malignant tumours (Kelly et al., 2011). Investigation into rapamycin's molecular mechanisms found that although rapamycin does not directly interact with mTOR, it does bind to the FK506-binding protein (FKBP-12) to form a complex that inhibits mTOR activity by binding to mTORC1 (Siekierka et al., 1989). Deforolimus, an analogue of rapamycin, is an inhibitor of mTOR which has been evaluated in a clinical setting. It was delivered via intravenous infusion daily for 5 days in a phase I clinical trial in solid tumours and haematological malignancies, positive responses were seen in patients with a variety of tumour types (Mita et al., 2008).

The PIM family of kinases has recently been shown to possess oncogenic and survival promoting properties (Nawijn et al., 2011; Shah et al., 2008). Inhibitors of this family of kinases, specific inhibition of PIM1 in particular, have been shown to induce apoptosis in human and mouse tumour cells in preclinical models (Hu et al., 2009). Other PIM1 inhibitors, such as ETP-45299 and SGI-1776, have been shown to inhibit cell proliferation and migration and influence apoptosis in tumours by resensitising them to conventional therapeutic treatments (Blanco-Aparicio et al., 2011; Chen et al., 2011c; Mahalingam et al., 2011; Mumenthaler et al., 2009).

Ruxolitinib, a janus kinase (JAK) inhibitor, has recently been approved for the treatment of myeloproliferative neoplasms (Mesa, 2010). The effect this inhibitor has is to selectively inhibit JAK1 and JAK2 kinases, whose role in certain myeloid and lymphoid cancers is to transduce extracellular signals to the nucleus via STAT (signal transducer and activator of transcription) proteins contributing to cell proliferation and differentiation (Eghtedar et al., 2012). Ruxolitinib inhibits JAK signalling via the cytokines that support transformed cells, arresting growth factor signalling by inhibiting downstream STAT phosphorylation. A phase II clinical study found that out of 18 patients with postmyeloproliferative neoplasm acute myeloid leukemia, 3 showed a significant response and 3 achieved complete remission, suggesting that ruxolitinib has a modest anti-leukaemic activity as a single agent (Eghtedar et al., 2012).

Rapidly accelerated fibrosarcoma (RAF) serine/threonine kinases are important signalling integrators comprised of 3 homologues, A-RAF, B-RAF and C-RAF (Bonner et al., 1984; Huleihel et al., 1986; Ikawa et al., 1988). Since the discovery of these kinases, the B-RAF gene was found to be mutated in a variety of different cancers leading to constitutive activation of the B-RAF kinase (Davies et al., 2002). Half of all melanomas exhibit this mutation and it is also found in a number of other cancers including colorectal and head and neck cancer (Pollock and Meltzer, 2002; Rajagopalan et al., 2002; Weber et al., 2003). An inhibitor of B-RAF, Vemurafenib, has successfully completed phase III clinical trials treating melanoma patients with a confirmed overall response rate greater than 50% and a median progression-free survival of 7 months. (Chapman et al., 2011).

### 1.4.2 Kinases with potential involvement in Apoptin phosphorylation

Phosphorylation is an important mechanism for the tumour specific function of Apoptin (Rohn et al., 2002). Studies have shown there are a number of potential Apoptin kinases that have been extensively investigated. This section will review the serine/threonine kinases PKC and PKG.

#### 1.4.2.1 Protein kinase C (PKC)

The serine/threonine protein kinase C (PKC) family of kinases were first identified in the early 1980s as the intracellular receptors for the tumour promoting phorbol esters (Castagna et al., 1982; Kikkawa et al., 1983). Currently, there are 12 known isoforms of PKC, the classical, the novel and the atypical isoforms (Mackay and Twelves, 2007). The classical isoforms include PKCα, PKCβ1, PKCβ2 and PKCγ, the novel isoforms are comprised of PKCδ, PKCε, PKCη, PKCμ and PKCθ, finally, the atypical isoforms are formed of PKCζ and PKCι/λ (Redig and Platanias, 2007).

All of the PKC isoforms share a common structure; a cell membrane targeting N-terminal regulatory domain linked by a flexible hinge region to the C-terminal catalytic domain (Newton, 2003; Parker and Murray-Rust, 2004) (Figure 1.4). The classical PKC isoforms regulatory domains, C1 and C2, are required for binding to the lipid second messenger diacylglycerol (DAG), phorbol esters and phosphatidylserine (PS) and also to $Ca^{2+}$. The novel isoforms share the C1 domain, but also contain a

novel C2 domain, which means that they are regulated by DAG but not $Ca^{2+}$. The C1 domain of the atypical isoforms of PKC, however, does not bind to DAG or $Ca^{2+}$ (Colon-Gonzalez and Kazanietz, 2006). The regulation of the atypical kinases is mainly controlled by protein-protein interactions mediated by the Phox/Bem1 domain (PB1) and the carboxy-terminal domain. Heat shock protein 90 (Hsp90) and mTORC2 are two proteins that have been shown to be involved in this process (Gould et al., 2009; Ikenoue et al., 2008).

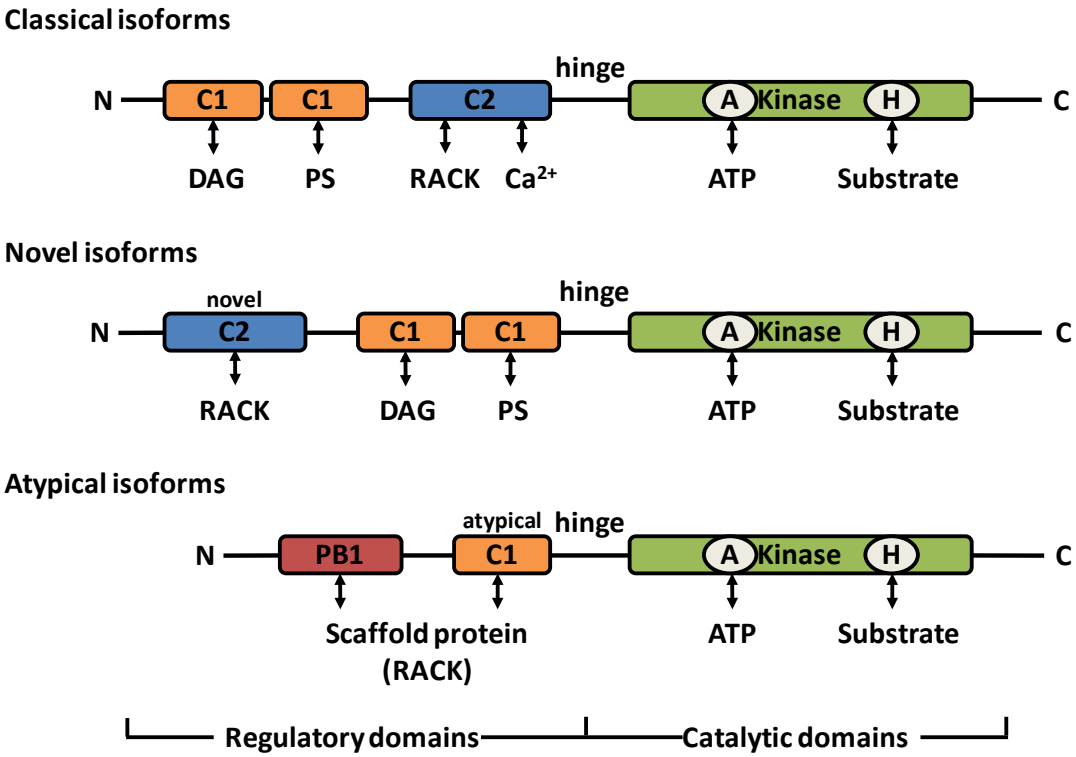



**Figure 1.4 Diagram of the structure of PKC isoforms.** The C1 domains are highlighted in orange, the C2 domains in blue, and the kinase domain in green. Arrows indicate interactions with labelled agents. A and H within the kinase domain are the activation loop and hydrophobic domain, respectively. DAG = diacylglycerol, PS = phosphatidylserine, ATP = adenotriphosphate, and RACK = receptor for activated C kinases. This figure was modified from another study (Newton, 2010).

Activation of the PKC isoforms involves recruitment to the plasma membrane. This is caused by stimulation of tyrosine kinase receptors and G-protein-coupled receptors which activate phospholipase C (PLC). PLC activation results in an increase in the level of DAG in the plasma membrane which, subsequently, induces relocalisation and activation of the PKC isoforms (Newton, 2003). However, in the case of atypical PKC isoforms, their activation is regulated by the phosphorylation of the hydrophobic motif by mTORC2 (Pearce et al., 2010). Binding of PKC to the plasma membrane causes a conformational change that exposes the binding sites of the kinase domain. Subsequent downstream events include the activation of the MEK-ERK and PI3K-Akt pathways (Balendran et al., 2000; Cai et al., 1997; Marshall, 1996).

Isoforms of PKC can localise differently inside the cell in response to apoptotic stimuli through intrinsic localisation sequences or specific-scaffolding protein binding (DeVries et al., 2002; Milani et al., 2003; Mirandola et al., 2004; Zauli et al., 1996). PKCδ, for example, translocates differently to the Golgi or mitochondrion, the nucleus or the membrane depending on its binding to ceramide, DAG or RACK (receptor for activated C kinases) (Steinberg, 2004), a protein with the ability to bind specific PKC isozymes in a manner that relieves their autoinhibition (Ron and Mochly-Rosen, 1995).

Studies show that the PKC isoforms have contrasting roles in tumour biology, with each isoform making a unique contribution to cancer formation and progression. For example, PKCε confers a growth advantage in transformed cells (Cacace et al., 1996), but PKCδ has been shown to possess anti-proliferative properties (Heit et al., 2001), with several isoforms of PKC having different, tissue-specific roles (Griner and Kazanietz, 2007). The variety of pathways activated by PKC isoforms dependent on sub-cellular localisation and access to specific substrates during activation explains why the PKCs possess such varied and contrasting properties in both normal and tumour cells (Wang et al., 1999).

In breast and colorectal cancers, PKCα has been shown to be over expressed and under expressed, respectively (Assert et al., 1999; O'Brian et al., 1989). Effects in normal cells have been reported to be anti-proliferative, with the activation of PKCα linked to G1 cell cycle arrest associated with ERK activation and induction of p21 and p27 (Frey et al., 1997). Studies indicate that modifications of PKCα are linked to regulation of cellular differentiation and the proliferation of a variety of cell types, CaCo-

2 and gastric epithelial cells being two examples (Scaglione-Sewell et al., 1998; Zhu et al., 1999).

PKCβ is separated into two spliced variants coded by the same gene, PKCβ1 and PKCβ2. These alternatively spliced variants are differentially involved in cell growth, apoptosis and transformation (Deacon et al., 1997; Ono et al., 1987; Zhu et al., 2000). PKCβ1 has been shown to function in the induction of survival in gastric cancer in response to chemotherapeutic agents (Jiang et al., 2002; Zhu et al., 2000). PKCβ2, by contrast, has been reported to lead to hyperproliferation and even to colon carcinogenesis in transgenic mice (Gokmen-Polar et al., 2001; Murray et al., 2002). The activation of PKCβ in tumour cells results in activation of Akt and glycogen synthase kinase 3β (GSK-3β), which leads to the transcription of vascular endothelial growth factor (VEGF), a molecule implicated in carcinoma growth (Graff et al., 2005).

PKCδ activation can have anti-apoptotic or pro-apoptotic effects (Wert and Palfrey, 2000), however, in many cell types, PKCδ is a critical pro-apoptotic kinase (Reyland, 2009). In pancreatic cancer, however, PKCδ is commonly overexpressed and activated compared to normal pancreatic tissue (El-Rayes et al., 2008). PKCδ has also been implicated in breast cancer, for example, oestrogen receptor positive breast cancer cell lines highly express PKCδ, while oestrogen receptor negative cell lines express reduced levels of PKCδ (Assender et al., 2007). In support of its pro-apoptotic properties, PKCδ has also been shown to down-regulate the expression of cyclins leading to cell cycle arrest (Black, 2000).

Follicular lymphoma and pancreatic cancer cells overexpress PKCζ when compared to non-malignant cells (Leseux et al., 2008; Peng et al., 2007). PKCε expression has been shown to decrease in breast cancer, pancreatic cancer and colon cancer in comparison to surrounding normal tissues (Doi et al., 1994; El-Rayes et al., 2008; Lavie et al., 1998). PKCε is associated with cell cycle arrest as well as differentiation, which could explain the reason for its downregulation in tumour cells (Nishizuka, 1984).

Increased expression of PKC or the differential activation of its isoforms has been linked to a variety of cancers including, but not limited to, breast, lung, thyroid, adenomatous pituitary cancers and leukaemias (Assender et al., 2007; Komada et al., 1991). As mentioned previously, PKC isoforms play a variety of cell type specific roles in the regulation of cell growth, apoptosis, and differentiation. Studies using RNA

interference techniques and genetic manipulation in mice of individual isoforms of PKC showed that isoforms α, β, λ, ε and ζ preferentially function to promote cell proliferation and survival, but PKCδ was found, in many cell types, to be pro-apoptotic (Reyland, 2009).

Taken together, the collected studies presented here highlight the observation that the expression of PKC isoforms appears to be responsible for the tumour specific characteristics observed in certain transformed cells and tissues compared to their normal counterparts.

### 1.4.2.2 Cyclic guanosine monophosphate-dependent protein kinase (PKG)

Cyclic guanosine 3', 5'-monophosphate (cGMP) is a ubiquitous second messenger that mediates several signal transduction pathways in mammalian cells. It is involved in the regulation of various physiological functions including neurotransmission and platelet aggregation (Fallahian et al., 2011). cGMP is also involved in the modulation of intracellular calcium levels in vascular smooth muscle cells (Eigenthaler et al., 1999; Smolenski et al., 1998; Vaandrager and de Jonge, 1996). cGMP has also been reported to play a role in cellular proliferation, cellular differentiation and apoptosis (Loweth et al., 1997; Shimojo et al., 1999). There are several intracellular targets of cGMP, specific phosphodiesterases (PDEs), cGMP-gated cation channels and cyclic guanosine monophosphate-dependent protein kinase (PKG) (Hofmann, 2005; Kaupp and Seifert, 2002; Torphy, 1998).

PKG belongs to the family of serine/threonine kinases and is comprised of three distinct isoforms that are transcribed from two genes (Orstavik et al., 1997; Orstavik et al., 1996). The PKG-I gene is alternatively spliced to form PKG-Iα and PKG-Iβ which are widely distributed but expression varies in different tissues, while PKG-II is more restricted to the brain, kidney and intestine.

PKG-Iα and PKG-Iβ differ only in the N-terminal ~100 amino acids (Francis et al., 2005; Francis and Corbin, 1999; Hofmann, 2005; Wernet et al., 1989). PKG-I monomers contain a regulatory domain towards the N-terminal portion of the protein and a catalytic domain towards the C-terminal portion. Each domain comprises subdomains that possess specific functions (Francis et al., 2005; Hofmann, 2005; Hofmann et al., 2009; Lincoln and Corbin, 1977; Monken and Gill, 1980). The

subdomains of the regulatory domain are the dimerisation and localisation subdomain provided by an extended leucine zipper motif, an autoinhibitory subdomain and a cGMP binding subdomain comprised of two homologous cGMP binding sites that are arranged in tandem. The two cGMP binding sites are called A and B, with A being a high affinity/slow dissociation binding site and B being a lower affinity/fast dissociation binding site (Kim et al., 2011). The catalytic domain contains two subdomains, a small lobe subdomain that binds to ATP and a large lobe subdomain that binds to protein substrates (Figure 1.5). The catalytic domain and the cGMP binding subdomains are conserved between the PKG-I isoforms (Richie-Jannetta et al., 2006).

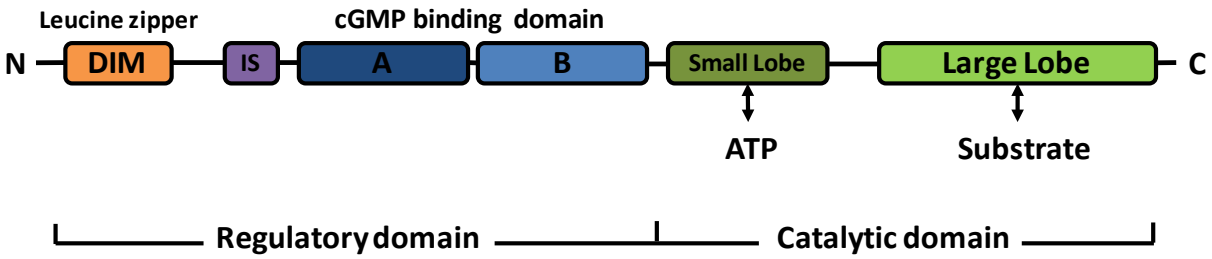



**Figure 1.5 Diagram of the structure of PKG isoforms.** The Leucine zipper dimerisation domain is highlighted in orange, the inhibitory subdomain in purple, the two cGMP binding sites: A (high affinity/slow dissociation) and B (lower affinity/fast dissociation) are highlighted in blue and the small lobe (ATP binding) and large lobe (protein substrate binding) of the catalytic domain are highlighted in green. ATP = adenotriphosphate and cGMP = cyclic guanosine monophosphate. This figure was modified from another study (Kim et al., 2011).

Specific substrates of PKG determine its physiological function through diverse signalling pathways (Ammendola et al., 2001; Schlossmann and Desch, 2009). Inositol triphosphate receptor-associated cGMP-kinase substrate (IRAG) is an endoplasmic reticulum-anchored membrane protein and a main signal transducer of PKG activity in the cardiovascular system (Schlossmann et al., 2000). PKG-Iβ stably associates with IRAG and phosphorylates this substrate at serine 696, this leads to suppression of

calcium release by the inositol 1,4,5-triphosphate receptor I ($IP_3RI$) in vascular and gastrointestinal smooth muscles and in platelets (Ammendola et al., 2001; Schlossmann et al., 2000). The phosphorylation of IRAG by PKG acts by inhibiting platelet aggregation, and reduced bleeding time reported in IRAG-KO mice suggests that this phosphorylation may play a role in blood clotting (Antl et al., 2007). In addition, the myosin phosphatase targeting subunit 1 (MYPT1) is phosphorylated by PKG-Iα at serine 695 resulting in an increase in phosphatase activity and relaxation in vascular smooth muscle cells.

Regulation of cellular membrane potential is partially governed by the calcium-activated maxi-$K^+$ channel ($BK_{Ca}$) and in vascular tissues, $BK_{Ca}$ regulates basal blood pressure (Sausbier et al., 2005). PKG phosphorylates $BK_{Ca}$ at serine 1134, and interestingly, phosphorylation by PKC at serine 1151 and 154 determines the activation of $BK_{Ca}$ by PKG (Zhou et al., 2010). Vasodilator-stimulated phosphoprotein (VASP) is involved in angiogenesis and the remodelling of the actin cytoskeleton in the cardiovascular system (Chen et al., 2008; Chen et al., 2004). PKG-I phosphorylates VASP at serine 239 in vascular smooth muscle cells which reduces the binding of VASP to actin and negatively affects the cells invasion and contraction of collagen matrices (Defawe et al., 2010). PKG also phosphorylates ras homolog gene family, member A (RhoA) at serine 188 resulting in inactivation of RhoA signalling which leads to the reduction of vascular remodelling and fibrosis in mouse blood vessels (Sawada et al., 2009). Other PKG substrates include the cysteine-rich protein 2 (CRP2) which is a protein phosphorylated by PKG-I that is involved in the neural processes of encoding and processing noxious stimuli in mice (Huber et al., 2000; Schmidtko et al., 2008). Phosphodiesterase 5 (PDE5) is another substrate phosphorylated by PKG, at serine 92, which is involved in the regulation of relaxation/contraction cycles of smooth muscle (Rybalkin et al., 2002).

PKG can be involved in a number of cellular processes depending on the cell type and the substrate that it interacts with and much of the above data describes kinase function in the cardiovascular environment. In relation to cellular proliferation and cancer, studies have shown that PKG-I levels decrease in primary cells as they are passaged during tissue culture and expression is lost in many cell lines (Cornwell and Lincoln, 1989; Lin et al., 2004b). The loss of PKG-I expression in cells as they become more proliferative, in the case of primary cell lines, could potentially indicate that PKG-I expression might be lost in the process of tumourigenesis. Many studies support this notion, for example, it was reported that PKG expression in normal and preneoplastic

ovarian surface epithelium was lost in neoplastic ovarian tumours (Wong et al., 2001). In addition, PKG-I expression has been shown to be greatly reduced in colon tumours when compared to normal colon epithelium (Hou et al., 2006). cDNA analysis discovered that PKG-I mRNA levels were dramatically reduced in several tumour types compared to their normal counterpart tissues. This study also showed that downregulation of PKG occurred at the transcriptional level in several cancer cell lines (Hou et al., 2006). Additionally, a recent study using quantitative real-time PCR (qRT-PCR) reported that PKG isoforms were downregulated in malignant and benign breast tumours when compared to respective normal tissues (Karami-Tehrani et al., 2012). Further supporting the notion of PKG loss during transformation, one study has shown that PKG expression is significantly reduced upon introduction of the simian virus 40 large T antigen (SV40LT), responsible for the transformation and immortalisation of primary cell lines (Fujii et al., 1995a).

Activation of PKG occurs when cellular cGMP levels increase in response to natriuretic peptides or nitric oxide (NO). Natriuretic peptides bind to membrane-spanning receptors that possess guanylyl-cyclase activity on the cytoplasmic side of the plasma membrane (Silberbach and Roberts, 2001). NO activates soluble guanylyl-cyclase, this is the more common mechanism by which cGMP levels are elevated (Hofmann et al., 2000). PKG has many effects on the expression of genes, and in smooth muscle cells (SMC) PKG is involved in the maintenance of differentiated and less proliferative populations of cells (Lincoln et al., 2006; Pilz and Broderick, 2005). PKG has also been shown to have anti-proliferative effects in cardiomyocytes and mesangial cells (Segawa et al., 2001; Shimojo et al., 1999). In addition, PKG expression was reported to inhibit the proliferation of gastric cancer cells through blocking epithelial growth factor (EGF) triggered MAPK signal transduction (Wu et al., 2012).

The widespread expression of PKG suggests that it may have a fundamental role in growth regulation, however, a number of studies report contrasting findings. In non-tumour cells such as a pancreatic β-cell line and human neutrophils, PKG was found to be pro-apoptotic (Brunetti et al., 2002; Loweth et al., 1997). However, in cell lines including cultured astrocytes, PC12 cells, adult rat contractile SMC and human endothelial cells, PKG was found to prevent apoptosis (Hood and Granger, 1998; Kim et al., 1999; Komalavilas et al., 1999; Takuma et al., 2001). More recently, PKG has been shown to induce apoptosis in breast cancer cell lines MCF-7 and MDA-MB-468 (Fallahian et al., 2012). The anti-tumour properties of PKG are also highlighted in its

ability to impair the adaptability of colon cancer cells to an anaerobic environment, or hypoxia, a vital requirement for rapid tumour growth in the absence of angiogenesis (Kwon et al., 2011).

Several mechanisms have been proposed for the apoptotic function of PKG, including upregulation of caspase genes, death receptors, JNK1 activation and attenuation of β-catenin (Huang et al., 2001; Soh et al., 2000; Soh et al., 2001; Thompson et al., 2000). In addition, another study indicated that the potential role of PKG in growth suppression in colon cancer could be through the suppression of the activity and reduction in the nuclear expression of oncogenic β-catenin (Whitt et al., 2012).

In the colon, growth factors of the Wnt family regulate the renewal of the luminal epithelium by the homeostatic control of the proliferation of colon cells. The Wnt family ligands work to stabilise the cellular levels of β-catenin in order to avoid uncontrolled proliferation. Disruption of the normal function of this pathway is thought to be the key step in initiating intestinal tumourigenesis (Giles et al., 2003). At increased protein levels, β-catenin interacts with lymphoid enhancer factor (LEF) and T-cell factor (TCF) transcription factors and enters the nucleus where it activates the expression of c-Myc, cyclin-D1 and c-Jun, cell growth related genes (Behrens, 2000; Lustig and Behrens, 2003). As cells advance through the luminal epithelium, the β-catenin in the cytoplasm associates with the adenomatous polyposis coli (APC) complex which leads to its phosphorylation by GSK-3β, causing ubiquitination and proteasomal degradation (Behrens, 2000). Many tumours of the colon express truncated forms of APC that do not bind to β-catenin, permitting the accumulation of excessive levels in the absence of Wnt (Chandra et al., 2012; Hanson and Miller, 2005).

The importance of β-catenin in intestinal tumourigenesis and the observations that PKG can inhibit the expression of β-catenin, indicate that PKG is an important factor in suppression of tumour characteristics, and potentially a normal cell specific protein kinase. This, in addition to the anti-cancer properties of PKG described here, provides evidence that PKG expression could act as a marker for tumour and normal cell identification, in specific cell and tissue types.

## *1.5 Objectives of this study*

Cancer is an aggressive disease that affects millions of people worldwide each year. A number of genetic and phenotypic changes are associated with the advancement of the disease, described as the hallmarks of cancer, which lead to the transformation of normal cells into malignant neoplasms. An important prerequisite of cancer therapies is a tumour selective property, the ability to preferentially target tumour or transformed cells for destruction, thus avoiding potentially dangerous side effects. Apoptin, a chicken anaemia virus derived protein, has been proven in many separate studies to possess a tumour selective cytotoxic function, highlighting its potential as a cancer therapeutic (Backendorf et al., 2008b; Noteborn, 2005; Tavassoli et al., 2005). Further investigation of Apoptin function revealed tumour specific modifications and localisation essential to its cytotoxic effects. Phosphorylation of Apoptin on threonine 108 (T108) was shown to be important for tumour specific function, and nuclear localisation in tumour cells was also shown to be a differentiating factor in Apoptin expression in normal and transformed cells (Rohn et al., 2002; Zhang et al., 2003).

Investigation into the phosphorylation of Apoptin on the T108 residue has so far failed to identify the kinase or kinases responsible. The main objective of this study was to further investigate the tumour specific function of Apoptin and attempt to identify potential kinases responsible for Apoptin phosphorylation and subsequent cytotoxicity. The specific aims of this study were as follows:

- Using the siRNA library targeting 54 serine/threonine kinases, identify specific kinases important for Apoptin cytotoxicity.
- Analyse expression of two candidate Apoptin kinases, PKG-I and PKCβ1, in a panel of paired normal and transformed cell lines.
- Investigate cell sensitivity to Apoptin in a panel of cancer cell lines in relation to the expression of PKCβ1 and PKG-I.
- Using siRNA knockdown and overexpression studies, analyse effects of these two kinases on Apoptin function.
- Investigate whether human gyrovirus Apoptin has similar function to CAV Apoptin.

The work presented here demonstrates a novel role for PKG-I and PKCβ1 in Apoptin function in normal and transformed cells. Results of the above objectives are explained in Chapters 3, 4, 5 and 6.

# Chapter 2

# Materials and Methods

## *2.1 Materials*

Plastics used in tissue culture were purchased from Greiner Bio-One. All chemicals used were obtained from Sigma, unless stated otherwise. Solutions and buffers were stored at room temperature unless specified. Cell culture media were stored at 4°C. 0.2 μm and 0.45 μm filters were purchased from Schleicher & Schuell.

### 2.1.1 Solutions, buffers and media

#### *Agarose gel*

A 1% (w/v) agarose solution was made in 100 ml Tris-acetate/EDTA (1× TAE) and boiled to dissolve the powder. The solution was cooled to 50ºC and ethidium bromide was added to a final concentration of 0.5 μg/ml. An electrophoresis tray was prepared by sealing the edges with tape and inserting a comb. The agarose solution was poured into the electrophoresis tray and the gel was left to set at room temperature. Prior to use, the tape and the comb were removed.

#### *Agarose gel electrophoresis loading buffer (10×)*

- 0.25% Orange G
- 20% (w/v) Ficoll 400
- 10 mM Tris pH8.0
- 10 mM $MgCl_2$
- Made up in $dH_2O$
- Stored at 4°C

#### *Ampicillin*

A stock solution of 50 mg/ml was made in $dH_2O$, sterilised by filtering through a 0.2 μm filter and stored at -20ºC. A 50 μg/ml working concentration was used.

#### *10% Ammonium persulphate (APS)*

The solution was made in $dH_2O$ and stored at 4ºC for two weeks.

### *Annexin V Binding buffer*

- 10 mM HEPES adjusted to pH 7.4
- 140 mM NaCl
- 2.5 mM $CaCl_2$

### *Annexin V – Allophycocyanin Conjugate (Invitrogen)*

The 250µl stock solution was diluted; 1µl of Annexin V in 100µl of Annexin V binding buffer.

### *Blocking buffer for indirect immunofluorescence cytochemistry*

3% (w/v) bovine serum albumin (BSA) in PBS

### *Blocking buffer for Western blotting*

5 or 10% (w/v) Marvel milk powder in TBST or 3% (w/v) BSA in TBST

### *Carbenicillin*

Carbenicillin solution (100 mg/ml) was purchased from Bioline and stored at -20ºC. A working concentration of 100 µg/ml was used.

### *Cell culture media*

**DMEM:**

- 10% or 15% FCS (tetracycline free FCS for PKG inducible HT29 clones), 50 µg/ml streptomycin, 100 µg/ml penicillin and 1 mM sodium pyruvate were added into 500ml of medium prior to use.
- Stored at 4ºC

**McCoy's 5A:**

- 10% FCS, 50 µg/ml streptomycin, 100 µg/ml penicillin and 1 mM sodium pyruvate were added into 500ml of medium prior to use.
- Stored at 4ºC

**Nut mix:**

- 4 mM L-glutamine
- 25 µg/L hydrocortisone
- 95% Nut mix medium (Gibco BRL 21331-020)
- 5% FCS, 50 µg/ml streptomycin, 100 µg/ml penicillin and 1 mM sodium pyruvate were supplemented into 500ml of medium prior to use.

- Stored at 4ºC

**RPMI:**

- 10% FCS (tetracycline free FCS was used for PKG inducible clones), 50 μg/ml streptomycin, 100 μg/ml penicillin and 1 mM sodium pyruvate were supplemented into 500ml of medium prior to use.
- Stored at 4ºC

**M3 Base A:**

- 10% FCS was supplemented into the medium prior to use.
- Stored at 4ºC

### *CellTiter-Glo® Reagent (Promega)*

- Purchased from Promega and stored at -20°C long term. For frequent use, solution was stored at 4°C for 48 hours with no loss in activity.

### *Coomassie Brilliant blue staining solution for Western Blotting*

- 0.25% Coomassie Brilliant blue R-250/G-250
- 45% methanol
- 44.75% water
- 10% acetic acid

### *Coomassie Brilliant blue destain*

- 45% methanol
- 45% water
- 10% acetic acid

### *Coumaric acid*

- 90 mM p-coumaric acid in DMSO (dimethyl sulfoxide)
- Stored at -20°C

### *Cycloheximide*

Cycloheximide solution (100 mg/ml) was purchased from Sigma and stored at 4ºC. A working concentration of 20 μg/ml was used.

### *Doxycycline*

Freshly prepared 10 mg/ml doxycycline solution was made in $dH_2O$ and stored at 4ºC. Before adding doxycycline to cultures, it was diluted 1/10 in DMEM and sterilised by filtering through a 0.2 μm filter. A working concentration of 2.5 μg/ml was used.

### *ECL buffer*

- 100 mM Tris-HCl (tris[hydroxymethyl]aminomethane hydrochloride) pH 8.5
- Stored at 4°C

### *EDTA solution (pH 8.0)*

A 0.5 M disodium ethylenediaminetetraacetate.$2H_2O$ (EDTA) was made in $dH_2O$. The pH was adjusted to 8.0 with NaOH. The solution was aliquoted and autoclaved.

### *Ethidium bromide*

A 10 mg/ml ethidium bromide solution was made in $dH_2O$ and stored at 4°C in the dark.

### *Freezing medium for cell culture*

- 10% DMSO
- 20% FCS
- 70% DMEM
- Stored at 4ºC

### *G418 (Invivogen)*

A stock solution of 100 mg/ml was purchased from Invivogen and stored at -20ºC. A working concentration of 500 μg/ml was used.

### *Hygromycin B (Invivogen)*

Hygromycin B solution (100 mg/ml) was purchased from Invivogen and stored at -20ºC. A working concentration of 250 μg/ml was used.

### *Laemmli Sample Buffer*

- 62 mM Tris base (2-amino-2(hydroxymethyl) propane-1,3,-diol (tris)) pH 6.8
- 10% glycerol

- 2% SDS
- 5% β-mercaptoethanol
- The buffer was made up in $dH_2O$ and stored at -20ºC.

Prior to use, the following components were added to 1 ml Laemmli Sample Buffer: aprotinin and leupeptin both at a final concentration of 1 μg/ml, phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 100 μg/ml and 3 μl of saturated bromophenol blue.

### *Luria-Bertani (LB) agar plates*

- 1.5% (w/v) agar in Luria-Bertani medium
- Sterilised by autoclaving
- Cool down to 50ºC
- Pour plates after the appropriate antibiotic was added to obtain the working concentration of 50 μg/ml.

### *Luria-Bertani (LB) medium*

- 1% NaCl
- 0.5% Bacto-yeast extract
- 1% Bacto-tryptone
- Made up in $dH_2O$ and the pH was adjusted to 7.0 with 5 M NaOH
- Sterilised by autoclaving

### *Luminol*

- 250 mM luminol in DMSO
- Stored at -20°C in the dark

### *Maxi and Midiprep solutions (QIAGEN)*

**Buffer P1 (Resuspension Buffer)**

- 50 mM Tris-HCl pH 8.0
- 10 mM EDTA
- 100 μg/ml RNase A
- Stored at 4ºC after addition of RNase A.

**Buffer P2 (Lysis Buffer)**

- 200 mM NaOH

- 1% SDS

**Buffer P3 (Neutralisation Buffer)**

- 3.0 M potassium acetate pH 5.5

**Buffer QBT (Equilibration Buffer)**

- 750 mM NaCl
- 50 mM MOPS pH 7.0
- 15% isopropanol
- 0.15% Triton® X-100

**Buffer QC (Wash Buffer)**

- 1.0 M NaCl
- 50 mM MOPS pH 7.0
- 15% isopropanol

**Buffer QF (Elution Buffer)**

- 1.25 M NaCl
- 50 mM Tris-HCl pH 8.5
- 15% isopropanol

### *Miniprep solutions (Promega)*

**Cell Lysis Solution (CLA)**

- 0.2 M NaOH
- 1% SDS

**Cells Resuspension Solution (CRA)**

- 5 mM Tris-HCl (pH 7.5)
- 10 mM EDTA
- 100 µg/ml RNase A

**Neutralization Solution (NSB)**

- 4.09 M guanidine hydrochloride
- 0.759 M potassium acetate
- 2.12 M glacial acetic acid

**Column Wash Solution (CWA)**

- 162.8 mM potassium acetate
- 22.6 mM Tris-HCl (pH 7.5)
- 0.109 mM EDTA (pH 8.0)

Add 95% ethanol to make a final concentration to be approximately 60% ethanol, 60 mM potassium actate, 8.3 mM Tris-HCl and 0.04 mM EDTA.

### *Mifepristone*

Mifepristone was stored at -20°C and a working concentration of 1-5 nM was prepared in DMEM prior to use.

### *MTT (3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) stock*

5 mg/ml MTT was prepared in PBS and sterilised by filtering through a 0.2 μm filter.

### *2% paraformaldehyde in PBS*

In order to prepare a 100 ml solution, 2 g paraformaldehyde was dissolved in 50 ml PBS, heated to 60ºC and mixed. A few drops of 5 M NaOH were added while mixing until the solution became clear, then PBS was added to a final volume of 100 ml. The solution was stored at 4ºC.

### *PBS (Phosphate-Buffered Saline)*

- 140 mM NaCl
- 2.7 mM KCl
- 8.0 mM $Na_2HPO_4$
- 1.5 mM $KH_2PO_4$
- Made up in $dH_2O$ and sterilised by autoclaving

### *500× Penicillin/Streptomycin stock solution*

- 1.25 g Streptomycin sulfate
- 2.5 g Penicillin G
- Made up in 50 ml $dH_2O$ and sterilised by filtering through a 0.2 μm filter

### *PMSF (Phenylmethylsulfonyl fluoride)*

10 mM PMSF was made up in isopropanol and stored at -20ºC.

### *Propidium iodide (PI) stock and working solutions*

Stock solution

- 1 mg/ml PI Stock solution: 1 mg PI was dissolved in 1 ml PBS and sterilised by filtering through a 0.2 μm filter. The solution was stored at 2º to 8ºC and protected from light for up to 1 month.

- 10 mg/ml Ribonuclease A Stock solution: The solution was stored at -20ºC

Working solutions

- In order to prepare 10 ml of working solution, 400 µl of PI stock solution and 500 µl of Ribonuclease A stock solution were added in 9100 µl PBS. The solution was sterilised by filtering through a 0.2 µm filter.

### *Puromycin*

Puromycin was purchased from Invivogen and stored at -20°C. A working solution of 0.2 µg/ml was used.

### *ReBlot Plus stripping buffer (Millipore)*

Reblot Plus 10x solution was diluted to 1x in $dH_2O$ and stored at 4°C and replaced after 5 uses, according to the manufacturer's instructions.

### *10× Running/Transfer buffer for Western blotting*

- 250 mM Tris base
- 2.5 M Glycine
- 1% SDS
- Made up in $dH_2O$

### *1× Running buffer for Western blotting*

- 10% 10× Running/Transfer buffer
- 90% $dH_2O$

### *10% SDS (sodium dodecyl sulphate)*

A 10% (w/v) solution of SDS was made up in $dH_2O$, heated to 68ºC to dissolve and the pH was adjusted to 7.2 with concentrated HCl.

### *SDS-Polyacrylamide gel*

30% acrylamide mix was purchased from AMRESCO®. TEMED (N,N,N',N',-tetramethyl-ethylenediamine) was purchased from Sigma.

**Resolving gel**

| | 6% | 8% | 10% | 12% |
|---|---|---|---|---|
| $dH_2O$ | 5.3 ml | 4.6 ml | 4.0 ml | 3.3 ml |
| 30% acrylamide mix | 2.0 ml | 2.7 ml | 3.3 ml | 4.0 ml |
| 1.5 M Tris pH 8.8 | 2.5 ml | 2.5 ml | 2.5 ml | 2.5 ml |
| 10% SDS | 0.1 ml | 0.1 ml | 0.1 ml | 0.1 ml |
| 10% APS | 0.1 ml | 0.1 ml | 0.1 ml | 0.1 ml |
| TEMED | 0.008 ml | 0.006 ml | 0.004 ml | 0.004 ml |
| Total | 10 ml | 10ml | 10 ml | 10 ml |

**Stacking gel**

- 2.7 ml $dH_2O$
- 0.67 ml 30% acrylamide mix
- 0.5 ml 1.0 M Tris pH 6.8
- 0.04 ml 10% SDS
- 0.04 ml 10% APS
- 0.004 ml TEMED

### *3 M sodium acetate (NaAc) pH 5.2*

A 3 M solution of sodium acetate (NaAc) was prepared in dH2O and the pH was adjusted to 5.2 with glacial acetic acid. The solution was aliquoted and autoclaved.

### *siRNA reagents*

siRNA control

- On-TARGET plus® control siRNA GAPD: Human (Thermo Scientific Dharmacon®), Cat# D-001830-01-05
- The targeting sequence: 5'-GUCAACGGAUUUGGUCGUA-3'

siRNA against PKG-I

- On-TARGET plus® SMARTpool PRKG1 (Thermo Scientific Dharmacon®), Cat# M-004658-04-0005
- The targeting sequences: GAACAAAGGCCAUGACAUU, GGACAGGACUCAUCAAGCA, GGAUUGACAUGAUAGAAUU, CCUAUAACAUCAUAUUGAG

siRNA against PKCβ1

- On-TARGET plus® SMARTpool PRKCB1 (Thermo Scientific Dharmacon®), Cat# M-003758-04-0005
- The targeting sequence: GAACCAAGGUCCCGGAAGA, GGAUGAAACUGACCGAUUU, CCAAGUCUAUGUCCAAGGA, AGAGUAAGGGCAUCAUUUA

siRNA library

- Human RTF SMARTpool serine threonine kinase siRNA Library (Thermo Scientific Dharmacon®), Cat# H-004400

### *Simply Blue Safe stain (Invitrogen)*

Simply Blue stain was added to gels for at least 1 hour and the gels were subsequently rinsed and washed with $dH_2O$, according to the manufacturer's instructions.

### *3 M sodium acetate (NaAc) pH 5.2*

A 3 M solution of sodium acetate (NaAc) was prepared in $dH_2O$ and the pH was adjusted to 5.2 with glacial acetic acid. The solution was aliquoted and autoclaved.

### *Solubilisation solution for MTT assay*

- 50% dimethylformamide
- 0.2% glacial acetic acid
- 20 mM HCl
- 20% SDS
- Made up in $dH_2O$

### *50× TAE (Tris-acetate/EDTA)*

- 242 g Tris base
- 57.1 ml glacial acetic acid
- 37.2 g $Na_2EDTA.2H_2O$
- Made up in $dH_2O$ to 1 litre and pH was adjusted to 8.5 and autoclaved.

### *10× TBS (Tris-Buffered Saline)*

- 250 mM Tris base
- 1.5 M NaCl
- made up in $dH_2O$, pH was adjusted to 7.4 using concentrated HCl and autoclaved.

### *TBST (Tris-Buffered Saline-Tween 20)*

0.05% Tween 20 in 1× TBS

### *TE (Tris-EDTA)*

- 10 mM Tris-HCl pH 8.0
- 1 mM EDTA pH 8.0
- Made up in $dH_2O$

### *1× Transfer buffer for Western blotting*

- 10% 10× running/transfer buffer
- 20% methanol
- 70% $dH_2O$

### *0.2% Triton X-100*

- 0.1 ml of Triton X-100
- 49.9 ml of PBS
- The solution was stored at room temperature

### *1× Trypsin*

- 10% 10× trypsin (T4549 Sigma)
- 90% versene

### *Versene*

- 0.270 mM EDTA pH 8.0
- Made up in PBS and sterilised by autoclaving

### *Zeocin*

Was purchased from Invivogen and stored at -20°C. A working concentration of 200 µg/ml was used.

### 2.1.2 Cell lines

***1BR3***

- Human normal fibroblasts
- Provider: Gift from Prof. Alan Lehmann, Sussex Centre for Genome Damage and Stability, University of Sussex, UK

***1BR3LT***

- Human SV40 large T-antigen transformed fibroblasts
- Provider: Gift from Prof. Alan Lehmann, Sussex Centre for Genome Damage and Stability, University of Sussex, UK

***HeLa***

- Human cervical carcinoma transformed with human papilloma virus (HPV) type 18E6/E7 genes
- Provider: American Type Culture Collection

***H357***

- HNSCC
- Gift from Prof. Stephen Prime, Department of Oral and Dental Science, University of Bristol, UK

***Saos-2***

- Human osteosarcoma
- Provider: American Type Culture Collection

***293A***

- Human primary embryonal kidney cells transformed with human adenovirus type 5 E1A and E1B, used for adenoviral packaging. Included in Ad-Easy adenovirus recombinant production kit (Qbiogene)

***AD-293***

- The AD-293 cell line is a derivative of the commonly used HEK293 cell line, with improved cell adherence and plaque formation properties. AD-293 cells, like HEK293 cells, produce the adenovirus E1 gene in trans, allowing the

production of infectious virus particles when cells are transfected with E1-deleted adenovirus vectors such as the pAdEasy™-1 vector. Standard HEK293 cells do not adhere well to tissue culture dishes, hindering adherent cell culture and plaque assay procedures. AD-293 cells demonstrate improved adherence to tissue culture dishes, making AD-293 cell monolayers less susceptible to disruption during cell passaging and plaque assays.

- Provider: Stratagene

### *HCT116*

- Human colorectal carcinoma
- Provider: Gift from Prof. Bert Vogelstein, The Howard Hughes Medical Institute and Sidney Kimmel Comprehensive Cancer Centre, Johns Hopkins University, Baltimore, MD

### *HCT116 PUMA$^{-/-}$*

- PUMA gene knockout in human colorectal carcinoma
- Provider: Gift from Prof. Bert Vogelstein, The Howard Hughes Medical Institute and Sidney Kimmel Comprehensive Cancer Centre, Johns Hopkins University, Baltimore, MD

### *HCT116 p53$^{-/-}$*

- p53 knockout in human colorectal carcinoma
- Provider: Gift from Prof. Bert Vogelstein, The Howard Hughes Medical Institute and Sidney Kimmel Comprehensive Cancer Centre, Johns Hopkins University, Baltimore, MD

### *HCT116 p53$^{+/-}$*

- p53 Heterozygous knockout in human colorectal carcinoma
- Provider: Gift from Prof. Bert Vogelstein, The Howard Hughes Medical Institute and Sidney Kimmel Comprehensive Cancer Centre, Johns Hopkins University, Baltimore, MD

### *HCT116 p300$^{-/-}$Clone D10*

- p300 knockout clone D10 in human colorectal carcinoma cells

- Provider: Gift from Prof. Carlos Caldas, Department of Oncology, Cancer Genomics Program, Hutchison/MRC Research Centre, University of Cambridge, Cambridge, UK

### *NCM460*

- Normal derived human colon cell line
- Provider: INCELL Corporation, LLC, 12734 Cimarron Path, San Antonio, TX 78249, USA

### *SW480*

- Human colon adenocarcinoma cell line
- Provider: Gift from Dr. Darren Browning, Department of Biochemistry and Molecular Biology, Molecular Oncology Program, MCG-CC Medical College of Georgia, 1120 15th Street, CB2605, Augusta, GA 30809-2100

### *SW480 mifepristone inducible clone J5B*

- Mifepristone inducible for PKG1α expression colon carcinoma cell line
- Maintenance of transgenes using 200 µg/ml Hygromycin B and 200 µg/ml Zeocin
- Provider: Gift from Dr. Darren Browning, Department of Biochemistry and Molecular Biology, Molecular Oncology Program, MCG-CC Medical College of Georgia, 1120 15th Street, CB2605, Augusta, GA 30809-2100

### *HT29*

- Human colon adenocarcinoma cell line
- Provider: Gift from Dr. Darren Browning, Department of Biochemistry and Molecular Biology, Molecular Oncology Program, MCG-CC Medical College of Georgia, 1120 15th Street, CB2605, Augusta, GA 30809-2100

### *HT29 doxycycline inducible clone 1 beta # 7*

- Doxycycline inducible for PKG1β expression colon carcinoma cell line
- Maintenance of transgenes using 0.2 µg/ml Puromycin and 200 µg/ml G418
- Provider: Gift from Dr. Darren Browning, Department of Biochemistry and Molecular Biology, Molecular Oncology Program, MCG-CC Medical College of Georgia, 1120 15th Street, CB2605, Augusta, GA 30809-2100

### 2.1.3 Plasmid constructs

**Expression plasmids**

***pBabe-puro***

- Dr. Joop Gäken, Department of Haematological Medicine, King's College London, UK

***pBabe-puro PKCβ1***

- Dr. Joop Gäken, Department of Haematological Medicine, King's College London, UK

***pcDNA$_3$ Flag-tagged Apoptin***

- Prof. Mathieu H.M. Noteborn, Biological Chemistry, Leiden University, Leiden, The Netherlands

***pcDNA$_3$ 3X Flag-tagged Apoptin***

- Assistant Prof. Jose G. Teodoro, Rosalind and Morris Goodman Cancer Center and Department of Biochemistry, Montreal, Quebec, Canada

***pcDNA$_3$ 3X Flag-tagged Human Gyrovirus Apoptin***

- Dr. Joop Gäken, Department of Haematological Medicine, King's College London, UK

***pcDNA$_3$ PKG1α***

- Gift from Dr. Darren Browning, Department of Biochemistry and Molecular Biology, Molecular Oncology Program, MCG-CC Medical College of Georgia, 1120 15th Street, CB2605, Augusta, GA 30809-2100

***pCMV-Apoptin***

- Dr. Lars Guelen, Head and Neck Oncology Group, King's College London Dental Institute, UK

### *pCMV-GFP-Apoptin*

- Dr. Lars Guelen, Head and Neck Oncology Group, King's College London Dental Institute, UK

### *pEGFP-C1 (ClonTech)*

- Dr. Joop Gäken, Department of Haematological Medicine, King's College London, UK

### *pEGFP-C1 Human Gyrovirus Apoptin*

- Dr. Joop Gäken, Department of Haematological Medicine, King's College London, UK

## 2.1.4 Adenoviruses and Lentiviruses

**Ad-GFP-p53**: expressing p53 fused to GFP, obtained from Prof. Bert Vogelstein, The Howard Hughes Medical Institute and Sidney Kimmel Comprehensive Cancer Centre, Johns Hopkins University, Baltimore, MD

**Ad-GFP**: expressing GFP, obtained from Prof. Bert Vogelstein, The Howard Hughes Medical Institute and Sidney Kimmel Comprehensive Cancer Centre, Johns Hopkins University, Baltimore, MD

**Ad-Apoptin**: expressing Apoptin, produced by Dr. Patrayu Taebunpakul, Head and Neck Oncology Group, King's College London Dental Institute, UK

**Lenti-GFP**: expressing GFP, obtained from Dr. Jie Jiang, Department of Haematological Medicine, King's College London, UK

**Lenti-GFP-Apoptin**: expressing Apoptin fused to GFP, obtained from Dr. Jie Jiang, Department of Haematological Medicine, King's College London, UK

### 2.1.5 Antibodies

**Primary antibodies**

***β-actin***

- Mouse monoclonal anti-β-actin
- Provider: Sigma
- Dilution: 1:5000 (Western Blotting)

***Flag***

- Mouse monoclonal anti-Flag
- Provider: Sigma
- Dilution: 1:1000 (Western Blotting), 1 µg per 1 ml of cell lysate (Immunoprecipitation)

***GFP***

- Rabbit polyclonal anti-GFP
- Provider: Cell signalling
- Dilution: 1:1000 (Western Blotting)

***M30 cytodeath***

- Mouse monoclonal anti-Cytokeratin 18 neo-epitope M30
- Provider: Peviva
- Dilution: 1:200

***Non-specific to T108 Apoptin***

- Rabbit polyclonal non-specific to Apoptin T108 raised against the peptide H2N-SLITTTPSRPRTA-CONH2
- Provider: Eurogentec
- Dilution: 1:1000 (Western Blotting)

***p53***

- Mouse monoclonal anti-p53 clone DO-7
- Provider: Novacastra Laboratories
- Dilution: 1:1000 (Western Blotting)

### *p73*

- Mouse monoclonal anti-p73
- Provider: Abcam
- Dilution: 1:500 (Western Blotting)

### *PARP p85*

- Rabbit polyclonal anti-PARP p85 fragment clone G734A
- Provider: Promega
- Dilution: 1:2000 (Western Blotting)

### *Phospho-Apoptin T108*

- Rabbit polyclonal anti-phospho-Apoptin T108 raised against the peptide H2N-SLITTT(PO3H2)PSRPRTA-CONH2
- Provider: Eurogentec
- Dilution: 1:1000 (Western Blotting), 1:200 (Immunocytochemistry)

### *PKCβ1*

- Mouse monoclonal anti-PKCβ1
- Provider: Santa Cruz
- Dilution: 1:500 (Western Blotting)

### *PKCδ-C20*

- Rabbit polyclonal anti-PKCδ C-20
- Provider: Santa Cruz
- Dilution: 1:1000 (Western Blotting)

### *PKG1*

- Rabbit monoclonal anti-PKG1
- Provider: Cell Signaling
- Dilution: 1:1000 (Western Blotting)

### *Tubulin*

- Mouse monoclonal anti-tubulin
- Provider: Sigma

- Dilution: 1:5000 (Western Blotting)

## Secondary antibodies

### *FITC*

- Goat-anti-rabbit IgG (whole antibody) FITC conjugate
- Provider: Sigma
- Dilution: 1:100 (Immunocytochemistry)

### *FITC*

- Goat-anti-mouse IgG (whole antibody) FITC conjugate
- Provider: Sigma
- Dilution: 1:100 (Immunocytochemistry)

### *Horseradish peroxidase*

- Donkey-anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase
- Provider: Amersham Life Science
- Dilution: 1:2000 (Western Blotting)

### *Horseradish peroxidase*

- Goat-anti-mouse IgG secondary antibody conjugated to horseradish peroxidase
- Provider: Sigma
- Dilution: 1:4000 (Western Blotting)

### *Texas-Red*

- Horse-anti-mouse IgG (H+L)-Texas-Red
- Provider: Vector Laboratories
- Dilution: 1:100 (Immunocytochemistry)

## *2.2 Methods*

### 2.2.1 Transformation of plasmid DNA into chemically competent *E. coli*

1 µl of plasmid DNA was added to 45 µl of competent cells. The mixture was gently mixed and the cells were incubated 30 minutes on ice, 30 seconds at 42°C and again 2 minutes on ice. Then 400 µl of Luria-Bertani-medium (LB-medium) was added and the cells were incubated at 37°C for 45 minutes. The mixture was centrifuged 1 minute at 13000 RPM, 300 µl of supernatant was removed and the pellet was resuspended gently in the remaining supernatant. 50 µl of the suspension of cells was plated on an LB-agar plate containing the appropriate antibiotic. After 16 hours incubation at 37°C, a single clone was used to inoculate LB-medium for mini- or midiprep plasmid isolation.

### 2.2.2 Plasmid purification

#### 2.2.2.1 Wizard® Plus SV Minipreps DNA Purification System: Promega

Plasmid DNA was purified using DNA purification columns from a midiprep kit (Promega) as recommended by the manufacturer. The protocol is based on a modified alkaline lysis procedure.

#### 2.2.2.2 Plasmid midi- and maxiprep: QIAGEN plasmid kits

Plasmid DNA was purified using DNA purification columns from a midiprep kit (QIAGEN) as recommended by the manufacturer. The protocol is based on a modified alkaline lysis procedure.

### 2.2.3 Measuring plasmid DNA concentration

The concentration of plasmid DNA was determined using Nanodrop (Labtech International Ltd, East Sussex, UK). A pure DNA has an $OD_{260}/OD_{280}$ ratio of 2.0, acceptable range 1.8-2.0.

### 2.2.4 Glycerol stocks

Glycerol stocks were generated from bacterial cultures containing plasmid constructs and vectors. 800 µl bacterial culture, taken from bacterial midi- or maxicultures was added to 200 µl autoclaved glycerol (VWR International Ltd, Leicestershire, UK). The glycerol stocks were stored at -70°C and were used to re-start the growing of bacteria containing plasmid constructs and vectors. Glycerol stocks were streaked out on LB plates containing the appropriate antibiotic.

### 2.2.5 DNA precipitation

DNA was precipitated in an eppendorf tube by addition of $^1/_{10}$ volume 3.0 M sodium acetate (pH 5.2) and then 2 volumes 100% ethanol (Rathburn). The precipitation mixture was incubated for 30 minutes at -70°C or overnight at -20°C and subsequently centrifuged for 10 minutes at 13000 RPM in a cold centrifuge. The supernatant was removed without disturbing the DNA pellet. 70% ethanol was filled to the brim of the eppendorf and the tube was centrifuged for 6-7 minutes at 13000 RPM. The supernatant was again removed under the tissue culture hood. The pellet was air-dried and dissolved in the desired volume of TE under the tissue culture hood.

### 2.2.6 Agarose gel electrophoresis

Agarose gel was prepared by adding 1% (w/v) agarose to 100 ml 1× TAE, which was diluted in $dH_2O$ from a 50× concentrated stock solution. The agarose was dissolved by heating the mixture to boiling. After the agarose solution was cooled down to approximately 50°C, ethidium bromide was added to a final concentration of 0.5 µg/ml. The agarose solution was poured in an electrophoresis tray, of which the edges

were sealed with sticking tape. Combs were used to create slots. The gel was allowed to set at room temperature. Subsequently, combs and tape were removed. The gel was placed in the electrophoresis tank and covered with 1× TAE. DNA samples were prepared by addition of $^{1}/_{10}$ (v/v) 10× electrophoresis loading buffer. DNA molecular weight markers (New England Biolabs) were run in parallel with the DNA samples as a reference for DNA fragment sizes and concentrations. The gel was run at 100 V. DNA was visualised by placing the gel on a UV transilluminator. Pictures were taken by using a gel documentation system (Syngene).

### 2.2.7 Cell culture

#### 2.2.7.1 Cell maintenance

Saos-2, HeLa, HCT116 and 293A cell lines were cultured in DMEM supplemented with 10% FCS, 50 μg/ml streptomycin, 100 μg/ml penicillin and 1 mM sodium pyruvate.

H357 cell lines were grown in Nut mix supplemented with 5% FCS, 4 mM L-glutamine, 25 μg/L hydrocortisone, 50 μg/ml streptomycin, 100 μg/ml penicillin and 1 mM sodium pyruvate.

1BR3 and 1BR3LT cell lines were grown in DMEM containing 15% FCS, 50 μg/ml streptomycin, 100 μg/ml penicillin and 1 mM sodium pyruvate.

HCT116 cell lines were grown in McCoy's 5A containing 10% FCS, 50 μg/ml streptomycin, 100 μg/ml penicillin and 1 mM sodium pyruvate.

PKG1α inducible SW480 J5B cells were grown in RPMI medium with 10% FCS, zeocin 200 μg/ml, hygromycin B 200 μg/ml and induced with 2.5 μg/ml mifepristone for 24 hours.

PKG1β inducible HT29 1 beta #7 cells were grown in RPMI medium with 10% tetracycline free FCS, puromycin 0.2 μg/ml, G418 200 μg/ml and induced with 2.5 μg/ml doxycycline for 24 hours.

NCM356 and NCM460 cells were grown in INCELL propriety medium M3-Base A with 10% FCS.

Any derivative clones were cultured in the same medium as the parental cell line and all cells were grown at 37ºC and 5% $CO_2$.

### 2.2.7.2 Subculturing of cell lines

Cells were subcultured every 3 to 5 days. For a T75 culture flask, cells were washed once with 10 ml versene and incubated with 1 ml 1× trypsin at 37ºC for 1-5 minutes depending on the cell line. Once cells had become rounded, they were resuspended in 10 ml of medium containing 10% FCS in order to stop trypsinisation. Cells were then diluted as desired and plated in new tissue culture flasks.

### 2.2.7.3 Cryopreservation and thawing of cell lines

Cells (approximately $5\times10^6$ cells in a T75 tissue culture flask) were trypsinised as previously described and centrifuged for 5 minutes at 900 RPM. The supernatant was removed. The cell pellet was resuspended in 1 ml of appropriate freezing medium and transferred to a cryovial. The vial was placed in a -70ºC freezer (Vip series -86ºC, Sanyo) in a freezing container (Nalgene Cryoware) filled with isopropanol to moderate the cooling to a rate of 1ºC per minute. The cells were left at -70ºC for at least 24 hours before transferring to a liquid nitrogen (-196ºC) tank for long term storage. In order to thaw cells, a cryovial was removed from liquid nitrogen and quickly thawed at 37ºC. Cells were plated in a T75 flask with 10 ml prewarmed medium and left overnight at 37ºC and 5% $CO_2$. Medium was replaced the following day.

### 2.2.7.4 Counting cells

Cells in suspension were counted by using a haemocytometer (Weber Scientific International). The haemocytometer was assembled by placing a cover glass, provided by the manufacturer, on the grids of the counting chambers. When a cover glass is put on the slide, each square contains 0.1 μl of solution. Approximately 10 μl of cell suspension was pipetted into the counting chamber and the 4 outer squares were counted. The concentration of cells/ml was the total number of cells in the 4 squares divided by 4 and multiplied by $10^4$.

### 2.2.8 Western blotting

#### 2.2.8.1 Protein extraction

Medium was removed from the cells and the dish was washed once in cold PBS. Cells in a 100-mm dish were lysed by scraping in 400 μl Laemmli sample buffer with a sterile disposable cell scraper. Lysates were kept on ice until use or at -20ºC for long term storage. Lysates were passed through a 25G needle or homogenised using a motor pestle and boiled for 5 minutes before loading on gel.

#### 2.2.8.2 Coomassie brilliant blue staining

5 μl of lysate from each sample was resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was subsequently incubated with Coomassie Brilliant blue stain for a minimum of 4 hours on a rocking table. The gel was destained with Coomassie Brilliant blue destain for 24 hours on a rocking table. Coomassie Brilliant blue destain was changed every 2-4 hours during destaining. Proteins in the gel stain blue and could therefore be visualised in each lane. In order to load an equal amount of protein for Western blotting, the intensity of the blue stain in different samples was compared to one another.

#### 2.2.8.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Glass plates (included in Bio-Rad Mini-PROTEAN III Module) were first washed with water, rinsed with $dH_2O$ and cleaned with 70% ethanol. The gel-casting apparatus was set up according to the manufacturer's instructions (Bio-Rad Mini-PROTEAN III, 165-3301). The resolving polyacrylamide gel solution was prepared and poured. 200 μl of ethanol was added to flatten the top surface of the gel. The gel was allowed to set at room temperature and the ethanol was removed by washing with $dH_2O$ and soaking up the remaining liquid with a Whatman filter paper. The stacking gel was prepared, poured on top of the resolving gel and the gel comb was inserted. The comb was removed once the stacking gel had set and the gel was placed into the electrophoresis apparatus. The gel-tank was filled with 1× Running buffer and equal amounts of protein were loaded in each well. 4 μl of PageRuler™ Prestained Protein Ladder (Fermentas

Life Sciences) was run simultaneously with the samples to determine protein size. Gel electrophoresis was performed for 1.5-2 hours at 80 V.

#### 2.2.8.4 Protein transfer onto nitrocellulose membrane

Proteins were transferred onto a 0.45 μm pore nitrocellulose membrane (Schleicher & Schuell, BA85-401191). This procedure was performed in a tray filled with 1× Transfer buffer to ensure that no air bubbles were trapped between the gel and the membrane. A soft scouring pad was put onto the black side of a transfer cassette, followed by two pieces of 3mm filter paper (Whatman, 3030917), the gel, the nitrocellulose membrane and again two pieces of filter paper, all cut to the same size as the gel (generally 8.5 cm × 5 cm). A soft scouring pad was put on top of all, the transfer cassette was closed and placed in the transfer tank (Bio-Rad Mini-PROTEAN III) filled to the brim with 1× Transfer buffer. Transfer was performed at either 50 V for 2-3 hours at 4ºC or 40 mA overnight at room temperature.

#### 2.2.8.5 Probing

Blots were blocked for 30 minutes in 10% blocking buffer (Marvel milk powder in TBST) or overnight at 4ºC in 5% blocking buffer. Subsequently, blots were incubated for 1.5 hours in primary antibody, diluted in 10% blocking buffer, rinsed three times in TBST and washed in TBST for 15 minutes once and 5 minutes twice. Then blots were incubated for 1 hour in secondary antibody conjugated to horseradish peroxidase diluted in 10% blocking buffer. Finally, blots were rinsed three times in TBST and washed for 1 hour followed by two washes in TBST for 5 minutes.

For probing with rabbit non-specific to Apoptin T108 and anti-phospho-Apoptin T108 antibodies, blots were blocked for 1.5 hours in 3% BSA in TBST, followed by washing in TBST 10 minutes for three times. Blots were then incubated for 1.5 hours in either non-specific to Apoptin T108 or rabbit anti-phospho-Apoptin T108 antibody, diluted in 3% BSA in TBST, followed by washing in TBST 5 minutes for six times. Then the blots were incubated for 1 hour in anti-rabbit secondary antibody conjugated to horseradish peroxidase diluted in 3% BSA in TBST. Finally, blots were washed in TBST 20-30 minutes for three times.

All antibody incubations and wash steps were performed at room temperature on a rocking table. For a second probing procedure after ECL visualisation, the blot was stripped for 10 minutes in Reblot Plus Strong buffer (Millipore) and rinsed twice for 5 minutes in blocking solution.

#### 2.2.8.6 Enhanced chemiluminescence

Horseradish peroxidase-conjugated antibody was detected using the enhanced chemiluminescence (ECL) method. Prior to use, the reaction solution was prepared by mixing 3 μl 30% w/v $H_2O_2$, 25 μl coumaric acid and 50 μl luminol to 10 ml ECL buffer. The blot was incubated for 1 minute in the reaction solution, drained and then sealed in cling film (Saran wrap). A RX autoradiograph (Fuji) was exposed to the nitrocellulose membrane for 5 seconds to 20 minutes and subsequently developed.

### 2.2.9 Transgene expression

#### 2.2.9.1 Transient expression

Transfection was performed as recommended by the manufacturer (Invitrogen, Paisley, UK). For Western blot analysis, cells were plated at a density of $1\times10^5$ in a 12-well tissue culture plate and allowed to attach overnight. 1.6 μg DNA was added to OptiMEM to make 100 μl solution and mixed. 4 μl LipofectAMINE 2000 (Invitrogen) was added to 100 μl OptiMEM and mixed. The DNA solution was added to the lipofectamine solution, gently mixed and incubated at room temperature for 20 minutes and the transfection solution was added to the cells. Cells were transfected for 4 hours at 37ºC in air with 5% $CO_2$. Transfection solution was then removed, the cells washed once with complete medium and cultured in complete medium until analysis.

For immunofluorescence studies, cells were seeded in Falcon 8-well culture slides (Becton Dickinson, Oxford, UK) at a density of $5\times10^4$ per well and transfected at 50-80% confluency. 0.8 μg DNA was added to OptiMEM to make 50 μl solution and mixed. 2 μl LipofectAMINE 2000 was added to 50 μl OptiMEM and mixed. The DNA solution was added to the lipofectamine solution, gently mixed and incubated at room temperature for 20 minutes and the transfection solution was added to the cells. Cells were transfected for 5 hours at 37ºC in air with 5% $CO_2$. Transfection solution was then

removed, the cells washed once with complete medium and cultured in complete medium until fixation and staining.

#### 2.2.9.2 Adenoviral infection

##### 2.2.9.2.1 Amplification of adenovirus

Amplification of adenovirus was essentially performed as described by Graham and Prevec (1991). 293A cells were grown to 90% confluence (in order to obtain approximately $1\times10^7$ cells) in 100-mm-diameter petri-dishes in DMEM with all the supplements. Cells were infected with virus at a multiplicity of infection (MOI) of 1:1 in a minimal amount of medium. Supernatant saved from previously infected cells could also be used to infect cells. Cells were harvested when a complete cytopathic effect (CPE) was visible, with 80-100% of the cells were rounded and 10-20% were floating. Cells were lifted from the dish by pipetting gently and collected by centrifuging 10 minutes at 900 RPM. The supernatant was immediately stored at -70$^o$C for further use. The pellet and a few ml of supernatant above the pellet were mixed, snap-frozen in cryovials and quickly thawed three times and centrifuged 10 minutes at 13000 RPM at 4$^o$C. The supernatant containing viruses was carefully removed and either stored at -70$^o$C until use or used immediately for further infection of 293A cells.

##### 2.2.9.2.2 $TCID_{50}$ titration of adenovirus by CPE assay

The tissue culture infectious dose$_{50}$ ($TCID_{50}$) value is defined from the dilution of virus at which there will be a 50% probability of an infectious particle being present. 293A cells were plated in 96-well plates with $5\times10^4$ cells/well in DMEM containing 5% FCS. 24 hours later, the cells were infected with adenovirus in log dilutions in a range of $1\times10^{-3}$ – $1\times10^{-10}$ in DMEM without FCS. Each dilution was added to 10 wells in a row. As a control, DMEM without virus was added to the two outer columns of wells. 7 days after infection, the cells were examined under a light microscope for cytopathic effect (CPE). CPE could be detected in a well if the whole cell monolayer or a large area of it was lysed. The number of wells showing CPE was scored at each dilution from the highest dilution at which all the wells showed CPE downwards. No CPE was observed in the control wells. The $TCID_{50}$ value was calculated using the following formula based on the Spearman-Kärber method (Hierholzer and Killington, 1996):

$Log_{10}TCID_{50}$ = Highest dilution giving 100% CPE + 0.25-0.5 (total number of wells with CPE/10)

#### 2.2.9.2.3 Adenoviral infection

Cells were plated at a density of $1.9\times10^4$ cells in 100 µl per well in 96-well plates. After 18-24 hours, adenovirus was diluted in medium and 100 µl was added per well in order to infect cells with a MOI of 10 virus particles per cell. Cells were incubated at 37°C and 5% $CO_2$ and assayed at 24, 48 or 72 hours after infection.

### 2.2.10 siRNA and plasmid transfection by Nucleofection

siRNA or plasmid transfection was performed using Amaxa™ Nucleofector™ as recommended by the manufacturer (Lonza Biologics, Cambridge, UK). Cells were collected by trypsinisation and an aliquot of the cells was counted to determine cell density as previously described in Section 2.2.7. The required number of cells was centrifuged for 10 minutes at 100 g (IEC Central-4X, Bedfordshire, England). The supernatant was removed completely. The cell pellet was resuspended carefully in 100 µl of Nucleofector® Solution per sample. siRNA (100 nM siRNA control GAPDH or 100 nM siRNA against the chosen gene) was added in cell suspension. Cell/siRNA suspension was transferred into a certified cuvette. The cuvette was inserted into the Nucleofector® Cuvette Holder and the selected programme was applied (D-024 for Saos-2 cell line, D-032 for HCT116 cell line and U-023 for 1BR3) by pressing the X-button on the Nucleofector® I Device. Once the programme was completed, the cuvette was taken out of the holder and immediately 500 µl of the pre-equilibrated culture medium was added to the cuvette and the sample was gently transferred into the prepared 12 well plate (final volume 1 ml media per well). At 48 hours of siRNA transfection, cells were infected with either Ad-Apoptin or Ad-GFP or left untreated or treated with 10 µg/ml cisplatin.

### 2.2.11 siRNA library (Dharmacon)

HCT116 cells were seeded and treated as per manufacturer's instructions. Optimisation of reagents and conditions of culture were performed as described, with quantification of gene silencing analysed using the Quantigene branched DNA (Panomics, Affymetrix, Cat# SA-10001-01, SA-10003-01, SA-24984-08) method. Branched DNA assays require the use of DNA as a method of amplifying a signal. Probe set oligonucleotides bind a contiguous region of the target RNA and signal amplification is performed by sequential hybridisation of branched DNA to the probe sets. Addition of a chemilumigenic substrate generates a luminescent signal proportional to the amount of target RNA present in the sample. Protocols were provided by the manufacturer and followed exactly. Dharmafect transfection reagent 4 was used with a HCT116 cell seeding density of $5x10^3$ per well in 96-well library plates.

### 2.2.12 Immunoprecipitation protocol

Cells were transiently transfected with indicated plasmids using lipofectamine 2000 (Invitrogen) and were harvested 48 hours after transfection. Cells were washed twice with PBS and lysed on ice in a solution containing 50mM Tris-HCl (pH 8.0), 150 mM NaCl, 1mM EDTA (pH 8.0), 100 mM NaF, 10% glycerol, 1mM $MgCl_2$ , 1% Triton X-100 and complete protease inhibitors (Roche). Immunoprecipitations were performed by incubating whole-cell extracts with the indicated antibodies prebound to protein A or G-Agarose beads (Roche). Then the immunoprecipitates were mixed gently for 2 hours at 4°C, washed 4 times in lysis buffer and denatured in Laemmli buffer.

### 2.2.13 Indirect immunofluorescence staining

Cells were seeded in Falcon 8-well culture slides at a density of $5\times10^4$ cells/well (for transfection) or $1.9\times10^4$ cells/well (for infection). At 24 or 48 hours post-treatment, cells were washed in PBS and directly fixed in 2% paraformaldehyde for 30 minutes, washed three times in PBS and permeabilised in 0.2% Triton X-100 for 15 minutes at room temperature. After washing the cells three times in PBS, they were blocked for 30 minutes in 3% bovine serum albumin (BSA) in PBS and then incubated for 1 hour at room temperature in primary antibody, diluted in 3% BSA in PBS. The cells were washed twice in PBS and incubated for 1 hour at room temperature in secondary antibody, diluted in PBS. For detection of GFP, cells were fixed in 2% paraformaldehyde for 30 minutes and permeabilised in 0.2% Triton X-100 for 15

minutes at room temperature. Subsequently, cells were mounted in Vectashield mounting medium (Vector Laboratories, Peterborough, UK) containing 4', 6-diamidino-2-phenylindole (DAPI) to stain nuclei. Cells were visualised using an Olympus BX61 fluorescence microscope. Digital images were taken using Cell^F software (Olympus, Japan).

### 2.2.14 MTT cell proliferation assay

Cells were seeded in 96-well plates at a density of $5\times10^3$ cells per well. Cell viability was measured at different time points depending on the experiment. The total volume per well before adding the MTT solution did not exceed 100 µl. Briefly, 20 µl of 5 mg/ml MTT in PBS was added to each well. Cells were incubated with the MTT compound for 2-4 hours at 37°C and 5% $CO_2$ after which 150 µl of solubilisation solution was added to each well. The plates were incubated for a further 16-24 hours and the $OD_{595}$ was measured using a Bio-Rad model 3550 microplate reader.

### 2.2.15 CellTiter-Glo® viability assay (Promega)

Cells were seeded at $5\times10^3$ cells per 100 µl/well in 96-well plates. After 24 hours, adenovirus was diluted in medium and 100 µl was added per well in order to infect cells. Cells were incubated at 37ºC and 5% $CO_2$ and viability was measured at 24, 48 or 72 hours after infection. Equilibration of the sample plate at room temperature for 30 minutes was required, followed by addition of 100µl of premixed CellTiter-Glo® reagent. The contents were mixed for 2 minutes and the plate was incubated for 10 minutes at room temperature. The luminescence was then measured using a glomax 96-well luminometer (Promega).

### 2.2.16 Cell cycle analysis by flow cytometry

Cells were plated in 12 well plates and infected with adenovirus at a MOI of 40. The samples were collected at 24 and 48 hours post-infection. Cells were fixed in 70% ethanol for a minimum of 2 hours at -20 ºC. Before staining with PI, the cells were centrifuged for 10 min at 100 g (IEC Centra-4X, Bedfordshire, England), the ethanol was decanted and the cell pellet was suspended in PI FACS staining solution. The

samples were incubated at 37°C for 30 min then analysed on FACSCanto within 24 hours.

### 2.2.17 Cell death analysis by flow cytometry

Cells were plated in 12 well plates and transfected for 24 hours prior to infection with adenovirus at a MOI of 40. The samples were collected at 24, 48 and 72 hours post-infection. Cells were trypsinised and centrifuged for 10 min at 100 g (IEC Centra-4X, Bedfordshire, England); pellets were resuspended in 100 µl Annexin V buffer and incubated at room temperature for 20 min. At least 10 min prior to analysis, 5 µl of PI solution was added to each sample and 100 µl of Annexin V binding buffer was added. Analysis was then performed on FACSCanto within 30 min.

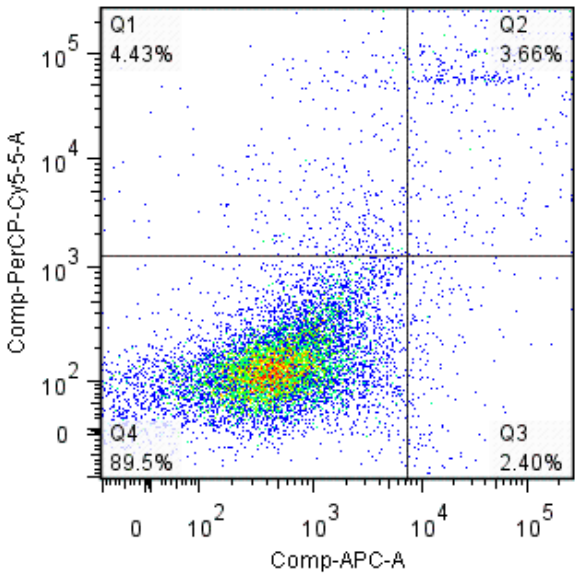



**Figure 2.1 FACS data analysis example graph.** Example analysis of FACS cell death data where Q1 is necrosis, Q2 is late apoptotis, Q3 is early apotosis and Q4 is live cells. PerCP-Cy5-5-a indicates levels of PI staining, and APC-A indicates levels of Annexin V staining. Cells with high levels of PI were considered to be dead or necrotic, cells with high levels of Annexin V were considered to be undergoing early stages of apoptosis and cells showing high levels of both dyes were marked as undergoing late apoptosis.

### 2.2.18 Statistical analyses

For statistical analyses, the student *t* test was carried out. Statistically significant difference was defined as $P<0.05$.

# Chapter 3

# Identification of the tumour specific Apoptin kinase/s

## *3.1 Introduction*

There are clear differences in Apoptin subcellular localisation between tumour and normal cells. Nuclear aggregation is observed in transformed cells whilst in normal cells, the protein is predominantly cytoplasmic (Guelen et al., 2004). The ability of Apoptin to translocate to the nucleus is due to the two nuclear localisation signals (NLS1 and NLS2) found in its basic structure (Danen-Van Oorschot et al., 1997).

In addition to the bipartite NLS, the 74-121 domain of Apoptin also contains a putative nuclear export sequence (NES), which suggests the ability to migrate not only to, but also from the nucleus (Poon et al., 2005). The C-terminal part of the protein contains a NLS with a phosphorylation site (threonine 108), that enables interaction with other proteins and modifications by kinases. This threonine phosphorylation is specific to tumour cells (Rohn et al., 2002; Rohn et al., 2005), where the protein localises to the nucleus and induces apoptosis. Phosphorylation of this threonine residue appears to be important for Apoptin cytotoxicity (Rohn et al., 2005). Substitution of threonine residue 108 with glutamic acid removed Apoptin's tumour specificity, causing it to aggregate in the nucleus of normal cells and induce apoptosis (Rohn et al., 2002; Zhuang et al., 1995b).

Importantly, phosphorylation of Apoptin on threonine 108 occurs specifically in tumours suggesting tumour specific kinase activity is involved in determining apoptotic response to the protein. Identification of such Apoptin kinase/s would therefore prove useful in developing greater understanding of Apoptin function and the mechanism of action, and could also lead to the development of cancer specific therapeutics.

To identify potential serine/threonine kinases for Apoptin phosphorylation, a siRNA library screen of 54 targets was used to knockdown kinases from the MAP kinase family. Cells were subsequently infected with an Apoptin expressing adenovirus and an MTT assay was performed to analyse any effects on Apoptin induced cell death.

## *3.2 Results*

### 3.2.1 Selection of a suitable cell line for a siRNA library screen for the identification of Apoptin kinase/s

The aim of these experiments was to determine variables for a human MAP kinase siRNA library, targeting 54 cell cycle checkpoint kinases known to have aberrant activity in cancer. This library was to be used in conjunction with Apoptin expression to identify which kinases play a role in Apoptin induced cytotoxicity. Specific factors were required for successful knockdown of the target genes and subsequent infection with Apoptin expressing adenovirus within a limited time span. A short treatment interval was important in order to measure the effect on Apoptin induced cytotoxicity, while ensuring the optimal knockdown of kinase expression by the siRNAs.

Initial studies investigated the current assays available for detecting changes in cellular viability. The MTT assay and Cell titre glo assay (Promega) were used in these experiments. The purpose of testing two viability assays was to determine the most sensitive measurement of Apoptin induced cell death. This, in combination with the siRNA library kinase knockdown, was used to ultimately attempt to detect the kinase/s involved in Apoptin cytotoxicity.

As a control, a chemotherapeutic drug (cisplatin) was added to Saos-2 cells and the cytotoxicity measured using MTT assay. The results (Figure 3.1) show that cisplatin efficiently kills Saos-2 cells when applied at concentrations of 20µg/ml or higher. At 24 hours, a loss of cellular viability of 40% was observed, and this result was similar using both assays (MTT and Cell titre glo). At a time point of 48 hours, the induction of cell death was further increased to 70%, again seen with both viability assays. From the results of the cisplatin treatment, the study investigated the sensitivity of a variety of cell lines towards Apoptin expression using adenoviral vectors. Saos-2 cells were tested first (Figure 3.2 and 3.3) and it could be seen that this particular cell line was not responsive to cell death induced by Apoptin in a short timeframe. At 72 hours with an MOI of 5-10, minimal cell death was observed (15-20%) when compared to the control. Another cell line, H357 cells, were infected with adenoviral and lentiviral vectors (Figure 3.4) and a drop in viability of 5-10% was evident when H357 cells were infected with MOI of 10 after 72 hours, with the result being the same in both assays.

As the H357 and Saos-2 cell lines were found to be resistant to Apoptin induced cytotoxicity within the required timepoint another cell line, HCT116, was used. The results in Figure 3.5 clearly show that, at an MOI of 20, HCT116 cells lost cellular viability by 25% after 24 hours post infection. After a longer time point of 48 hours, cell death had increased to almost 50%. The results described were similar using both viability assays, but cell titre glo detected 15% more killing after 48 hours than MTT. These results highlight the HCT116 cell line as a suitable candidate for use with the kinase siRNA library. The MTT assay was chosen for the library screen as it gave the most consistent and reliable results when compared to cell titre glo (Figure 3.3).

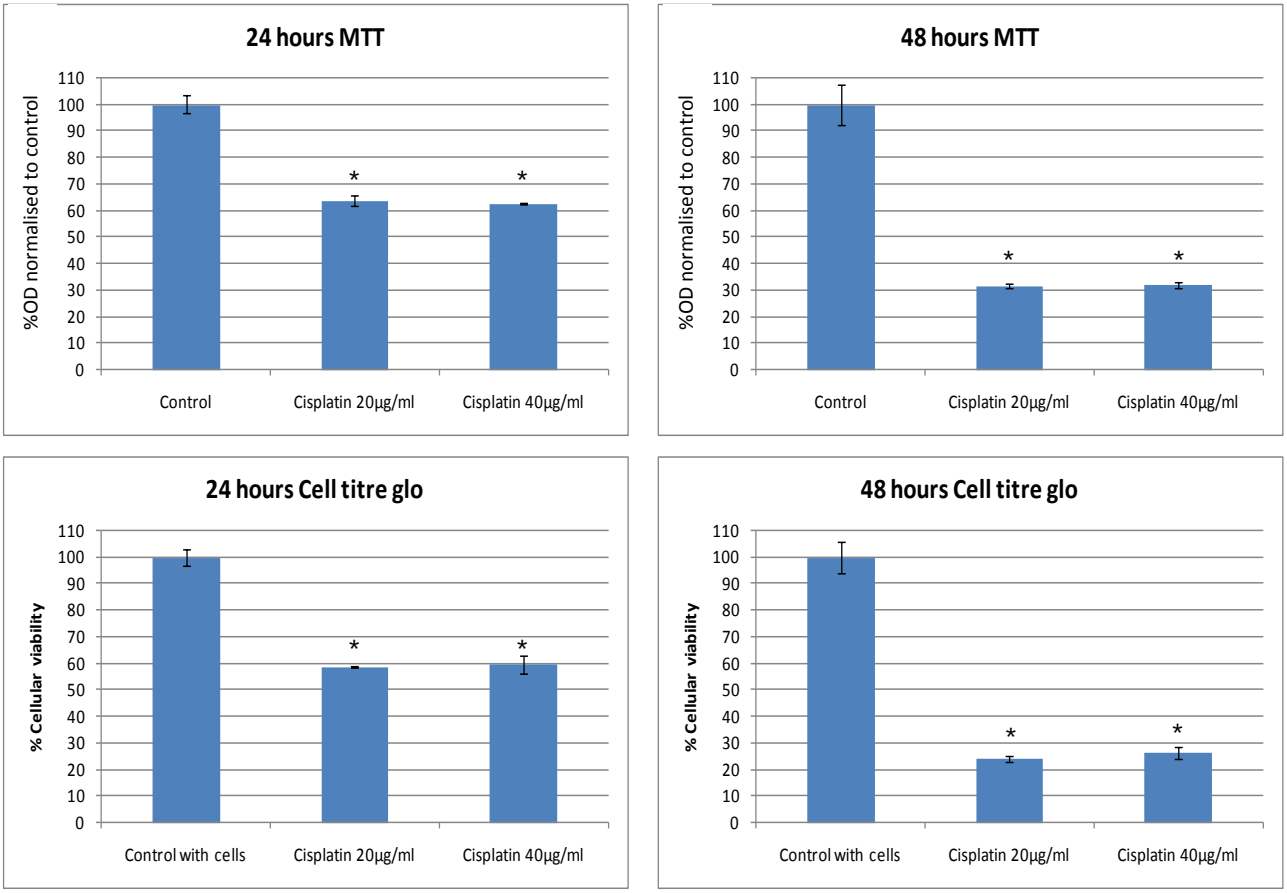



**Figure 3.1 Comparison of two viability assays.** MTT and cell titre glo assays comparing cellular killing of Saos-2 cells by varying concentrations of cisplatin. Normalised to control values. Error bars indicate standard deviation. n=3.

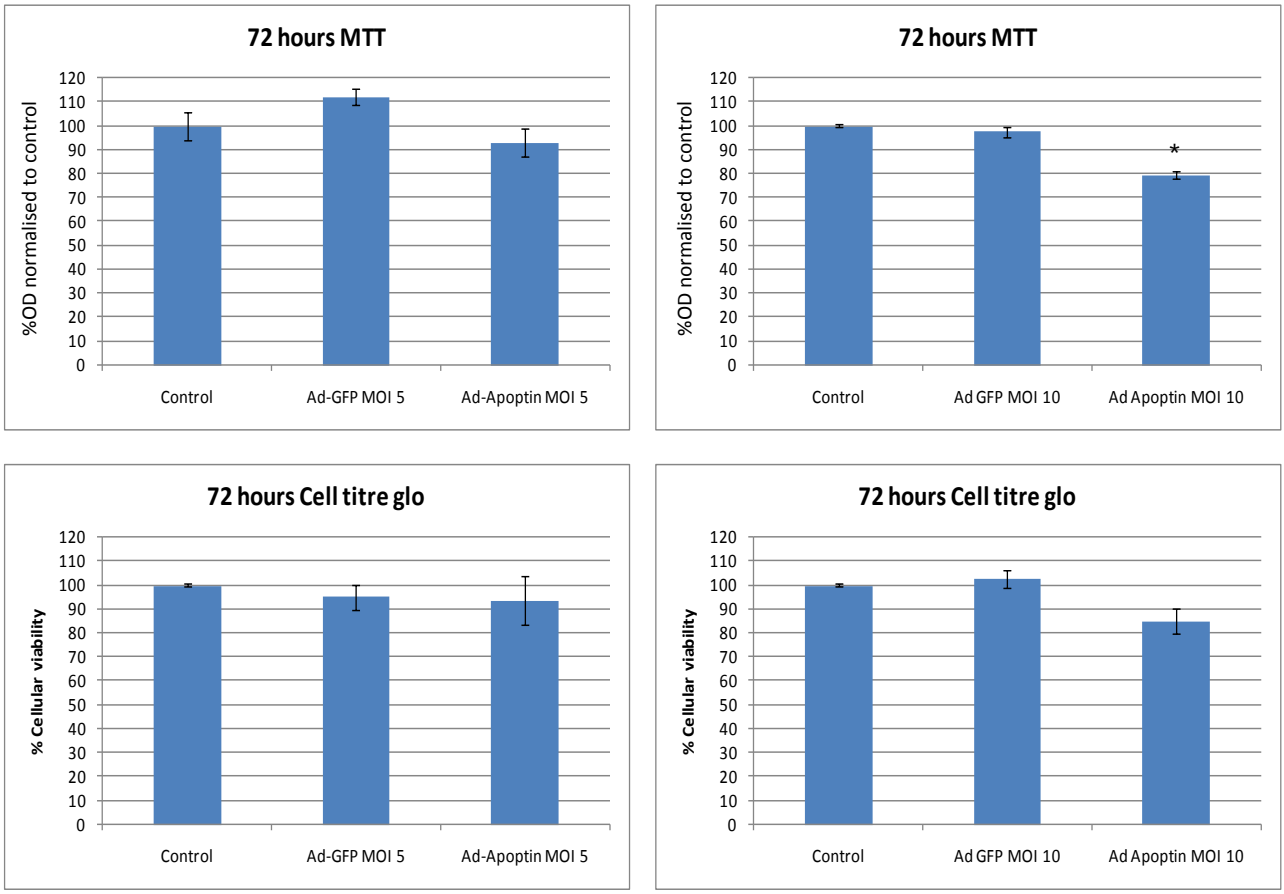



**Figure 3.2 Infection of Saos-2 cells with ad-Apoptin.** MTT analysis and cell titre glo results for Saos-2 cells infected with increasing MOI of adenoviral Apoptin at 72 hours post infection. Normalised to control values. Error bars indicate standard deviation. n=3.

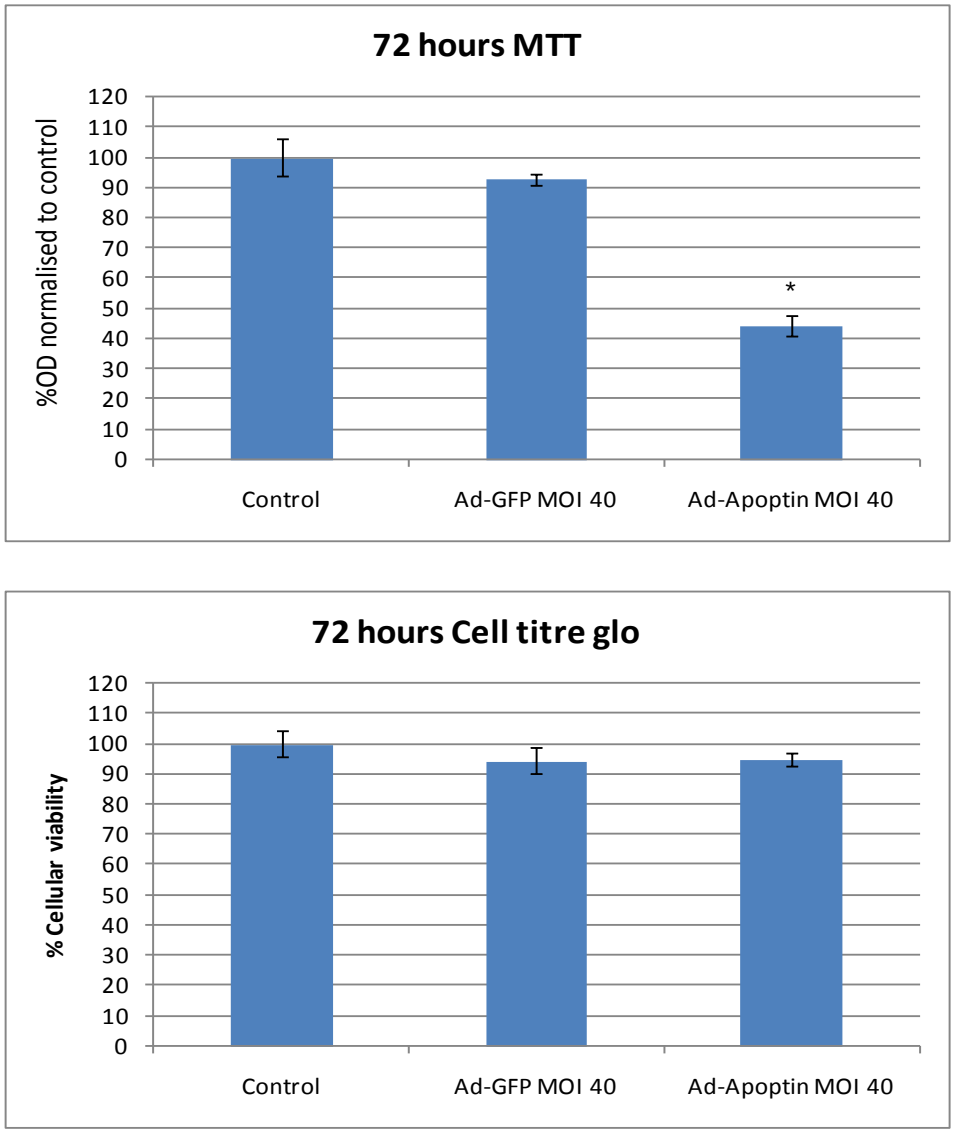
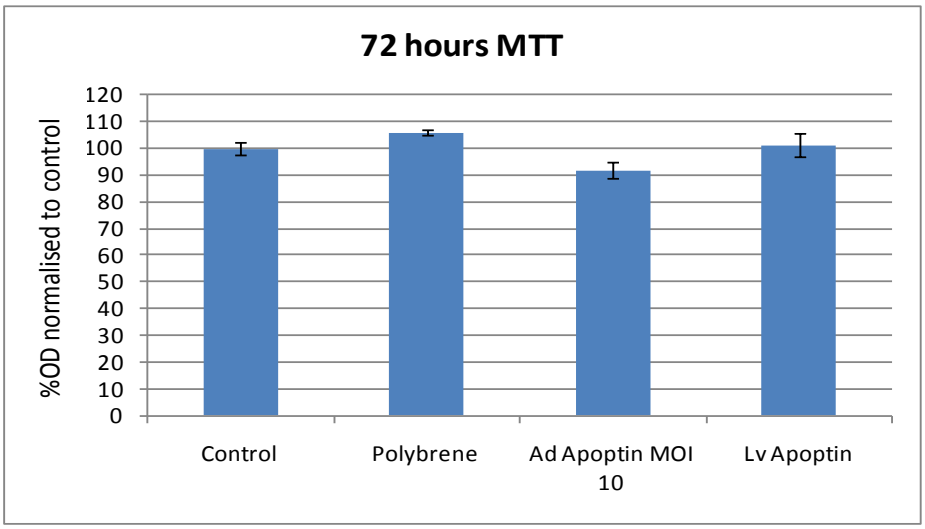



**Figure 3.3 Infection of Saos-2 cells with ad-Apoptin.** MTT analysis and cell titre glo results for Saos-2 cells infected with MOI 40 of adenoviral Apoptin at 72 hours post infection. Normalised to control values. Error bars indicate standard deviation. n=3.



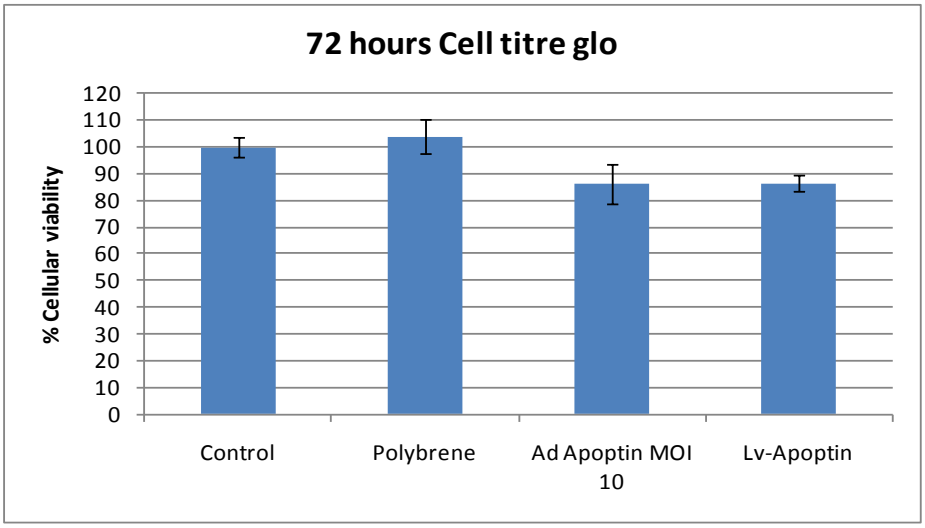




**Figure 3.4 Infection of H357 cells with adenoviral Apoptin and lentiviral Apoptin.** MTT analysis and cell titre glo results for H357 cells infected with MOI 10 of adenoviral Apoptin and MOI 8 of lentiviral Apoptin at 72 hours post infection. Normalised to control values. Error bars indicate standard deviation. n=3.

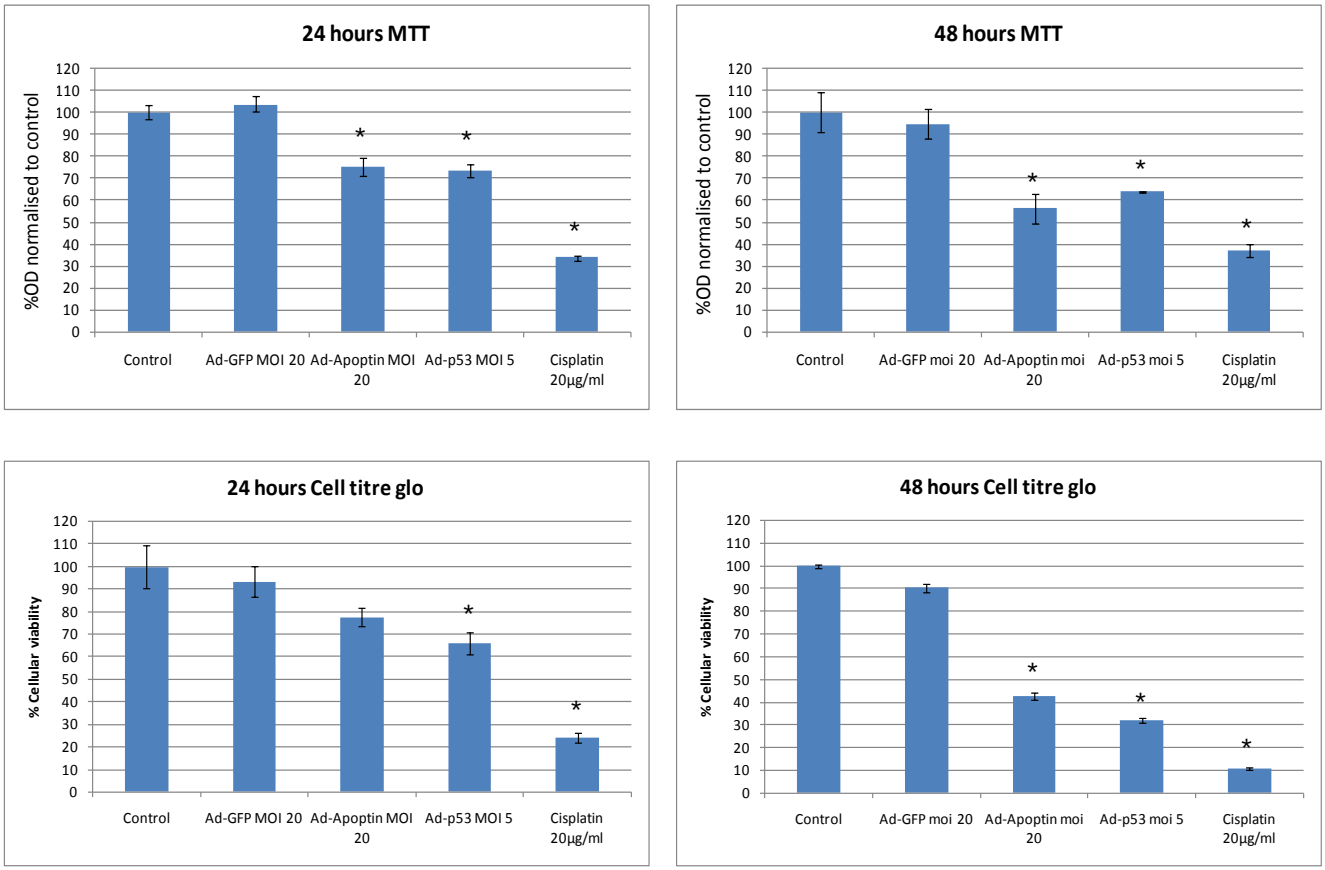



**Figure 3.5 Infection of HCT116 cells with adenoviral Apoptin.** MTT analysis and cell titre glo results for HCT116 cells infected with MOI 20 of adenoviral Apoptin at 24 and 48 hours post infection. Normalised to control values. Error bars indicate standard deviation. n=3.

### 3.2.2 Optimisation of reagents and siRNA controls for the siRNA library using HCT 116 cell line

In order to complete the siRNA library protocol, experimental design optimisation was required for the desired cell line. The first optimisation protocol determined the best transfection reagent and its optimum concentration from a panel of 4 separate Darmafect (DF) transfection reagents (DF1, DF2, DF3 and DF4) that would provide the highest transfection efficiency and the lowest cytotoxicity.

Using MTT assay, cellular viability was measured after 48 hours of treating the cells with 4 different transfection reagents at different concentrations. A control siRNA for GAPDH was used. To analyse the transfection efficiency and knockdown efficiency of the reagents, we used a branched DNA kit (Quantigene) from Panomics, Affymetrix that produces a luminescent signal when bound to the target DNA.

As shown in Figure 3.6A, the highest recorded knockdown of GAPDH, also induced a high level of cytotoxicity in the cells. In order to compromise the siRNA knockdown with the survival of the cells, a concentration providing the highest cellular viability and the greatest knockdown of the target gene was required. Analysis of Figures 3.6A and 3.6C, determined that 0.025µl of reagent 4 was sufficient to provide an efficient level of knockdown with minimal cytotoxicity.

Control siRNA for the siRNA library was chosen by knockdown of three separate housekeeping genes as the protocol for optimisation of siRNA library kit 2A recommends. Using a 96-well plate of siRNAs, siRNA efficiency was analysed using three genes at different cell densities and differing volumes of transfection reagent 4. Cells were once again incubated for 48 hours prior to knockdown analysis using the previously described Quantigene kit. Results show that at a low cell density and a reagent concentration of 0.025µl, GAPDH induced knockdown at the highest efficiency with minimal off target effects (Figure 3.7A) when compared to Lamin A/C (Figure 3.7B) and Cyclophilin B (Figure 3.7C).

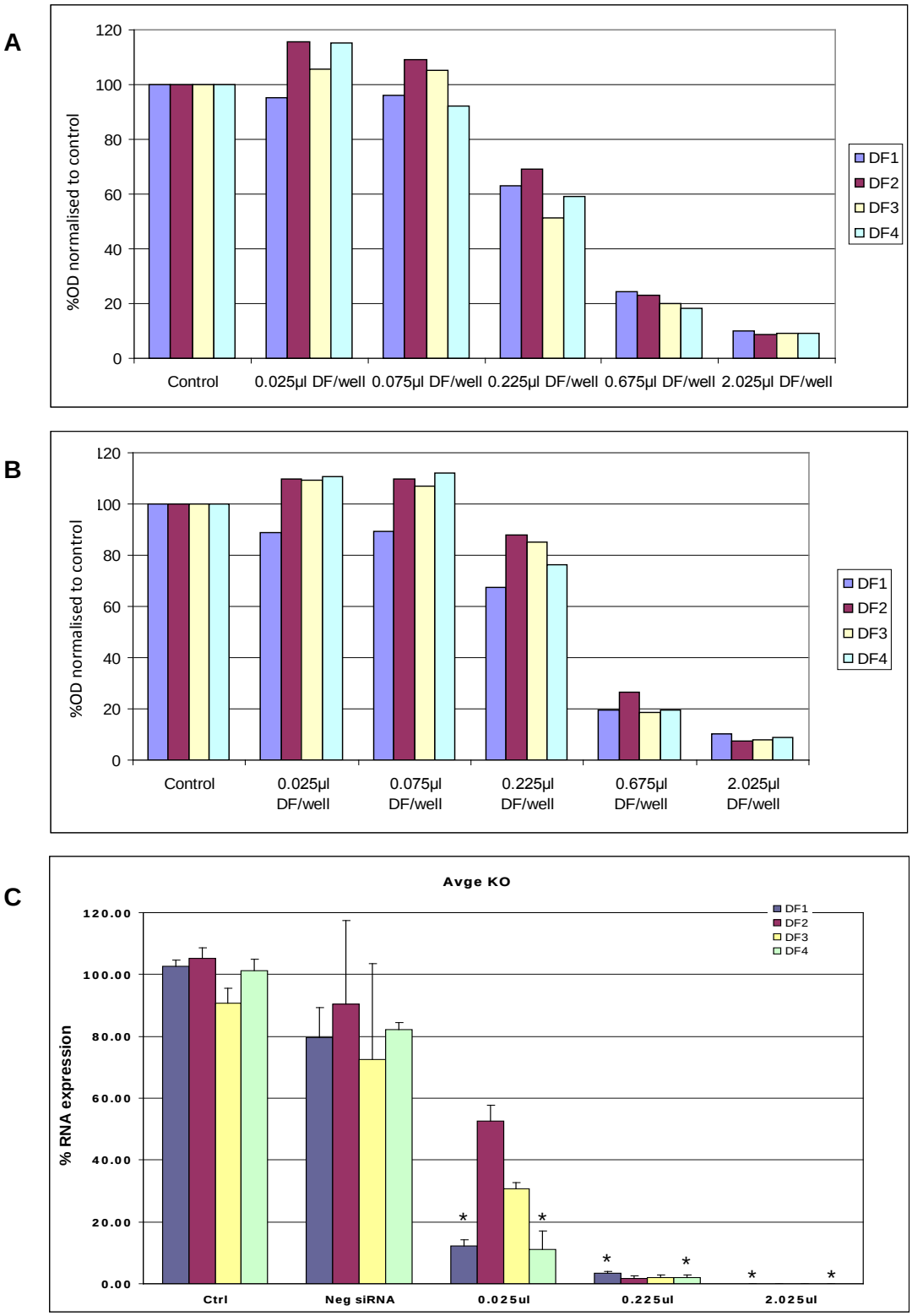



**Figure 3.6 Optimisation of HCT116 cells with increasing concentrations of 4 different transfection reagents.** MTT assays of HCT116 cells treated with control GAPDH siRNA (A) and negative control scrambled siRNA (B) showing cellular viability after 48 hours (n=1). Quantigene analysis of GAPDH knockdown by RNA expression (C). Normalised to control values. Error bars indicate standard deviation. DF1, DF2, DF3 and DF4 are the 4 different Darmafect transfection reagents used for optimisation.

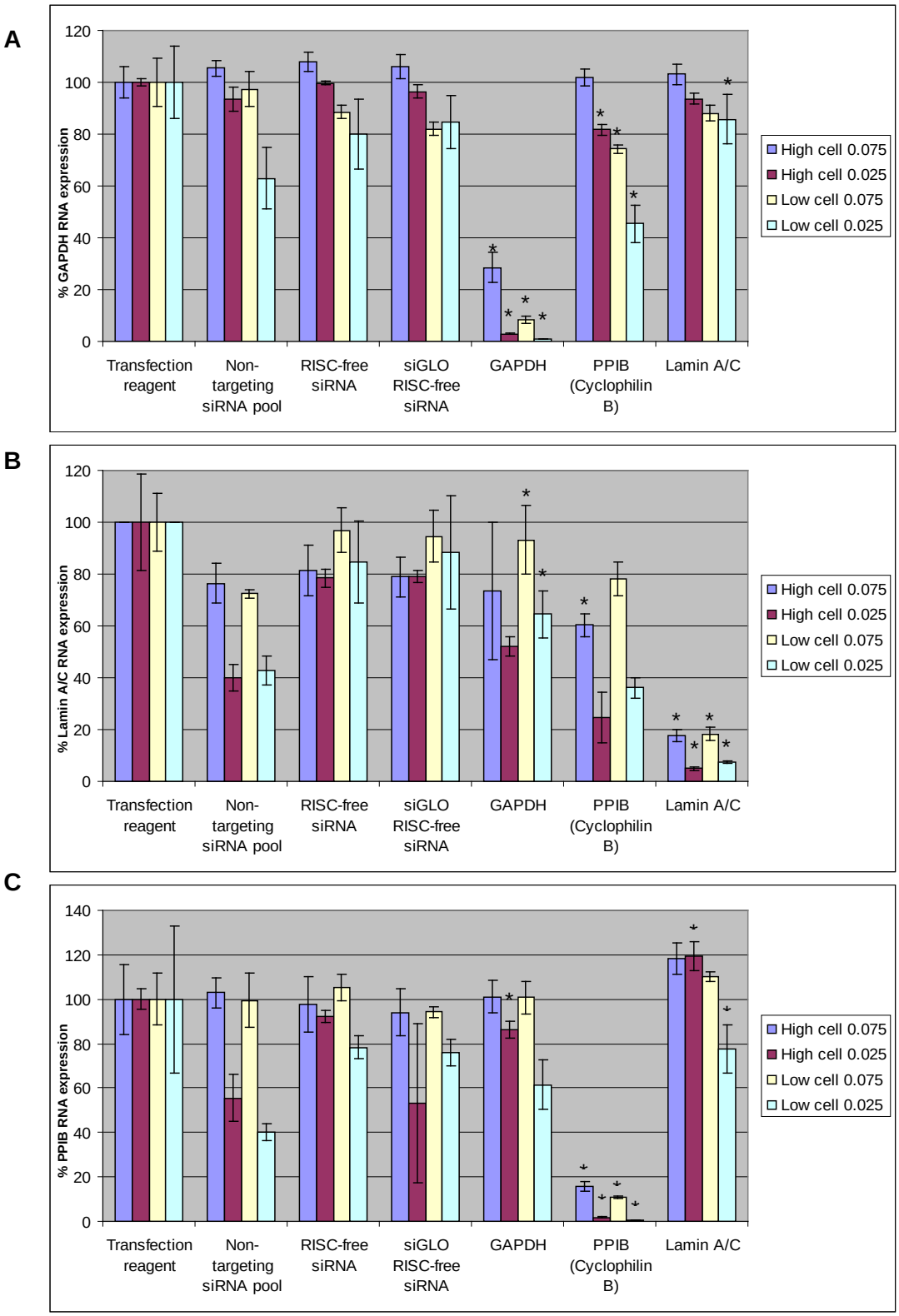
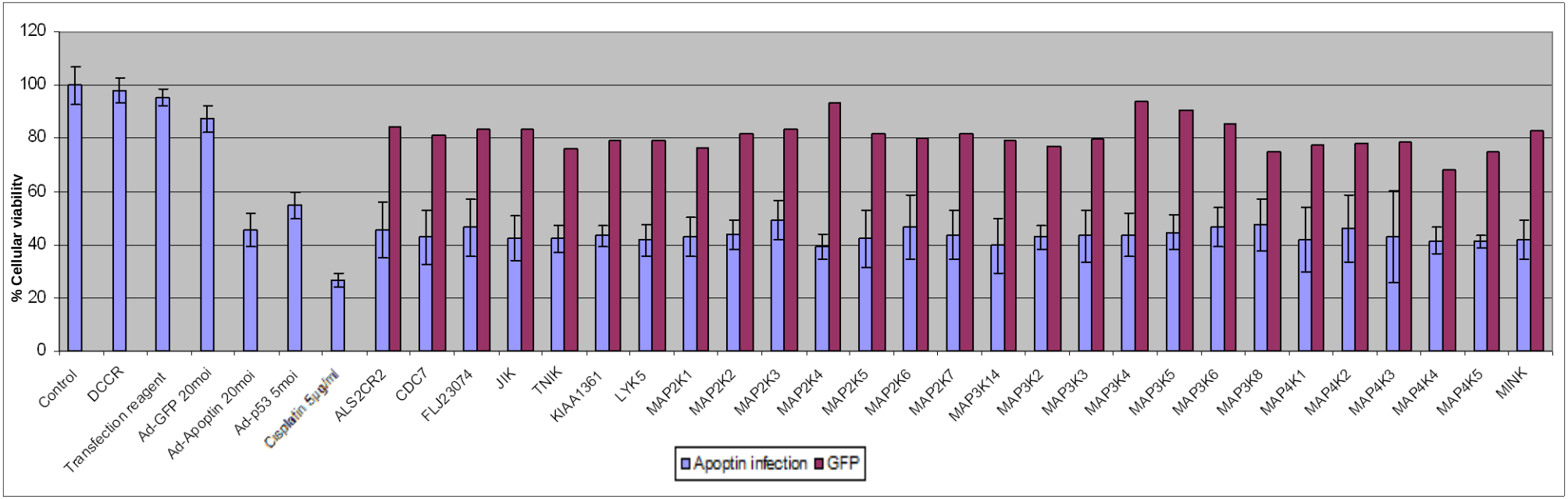



**Figure 3.7 Optimisation of HCT116 cells with control genes.** Quantigene analysis showing percentage knockdown of HCT116 cells exposed to siRNA against GAPDH (A), Lamin A/C (B) and Cyclophilin B (C). Variation in cell density (low cell density $5x10^3$, and high cell density $10x10^3$) and Darmafect reagent concentration were employed. Normalised to control values. Error bars indicate standard deviation. DF1, DF2, DF3 and DF4 are the different Darmafect transfection reagents used for optimisation.

### 3.2.3 Determination of kinase/s involved in Apoptin cytotoxicity

Once optimisation of the library controls and reagents was completed, the siRNA library screen was performed. MTT analysis of HCT116 cells infected with ad-Apoptin at MOI 20 for 24 hours showed that with the kinases targeted in this library, no significant difference in Apoptin cytotoxicity could be determined (Figure 3.8). Although variation is seen across the genes knocked down, no particular gene was found to have a significant positive or negative effect on Apoptin cytotoxicity.

**A**


% Cellular viability
120
100
80
60
40
20
0
Control
DCCR
Transfection reagent
Ad-GFP 20moi
Ad-Apoptin 20moi
Ad-p53 5moi
Cisplatin 5μg/ml
ALS2CR2
CDC7
FLJ23074
JIK
TNIK
KIAA1361
LYK5
MAP2K1
MAP2K2
MAP2K3
MAP2K4
MAP2K5
MAP2K6
MAP2K7
MAP3K14
MAP3K2
MAP3K3
MAP3K4
MAP3K5
MAP3K6
MAP3K8
MAP4K1
MAP4K2
MAP4K3
MAP4K4
MAP4K5
MINK
Apoptin infection
GFP

**B**

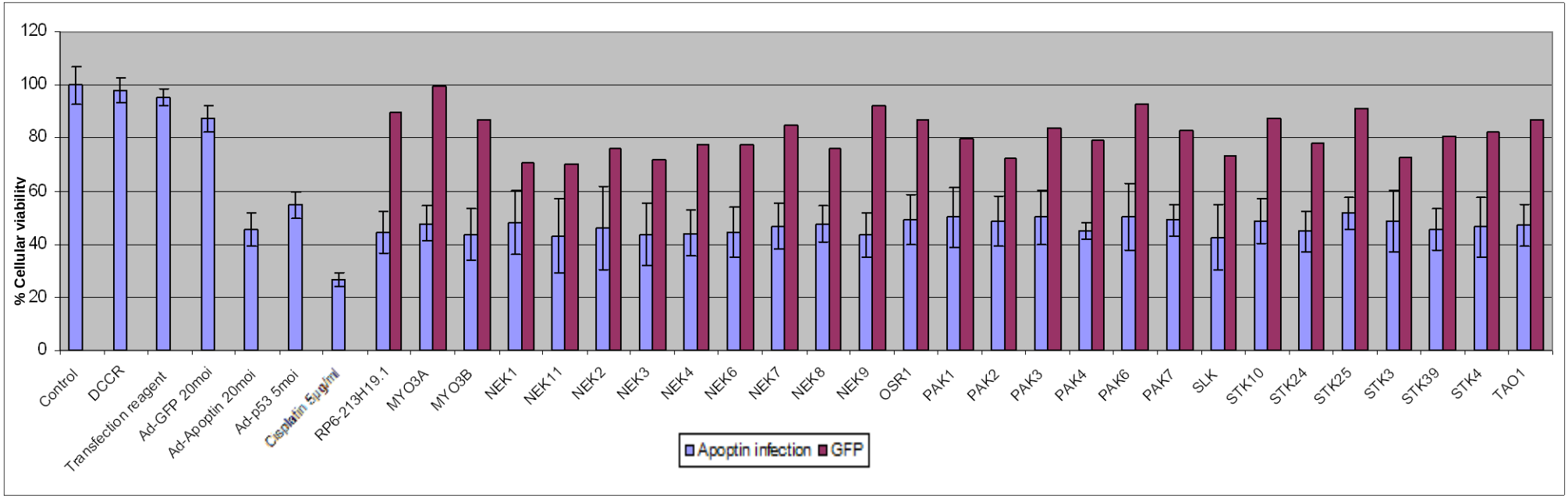



**Figure 3.8 Percentage cellular viability of HCT116 cell line after knockdown of various kinases.** MTT assay showing effect on apoptin cytotoxicity of knockdown of the kinases in the siRNA library. A = the first 27 kinases, B = the second 27 kinases of the library screen. The first 7 bars are controls. Normalised to control value. Error bars indicate standard deviation.

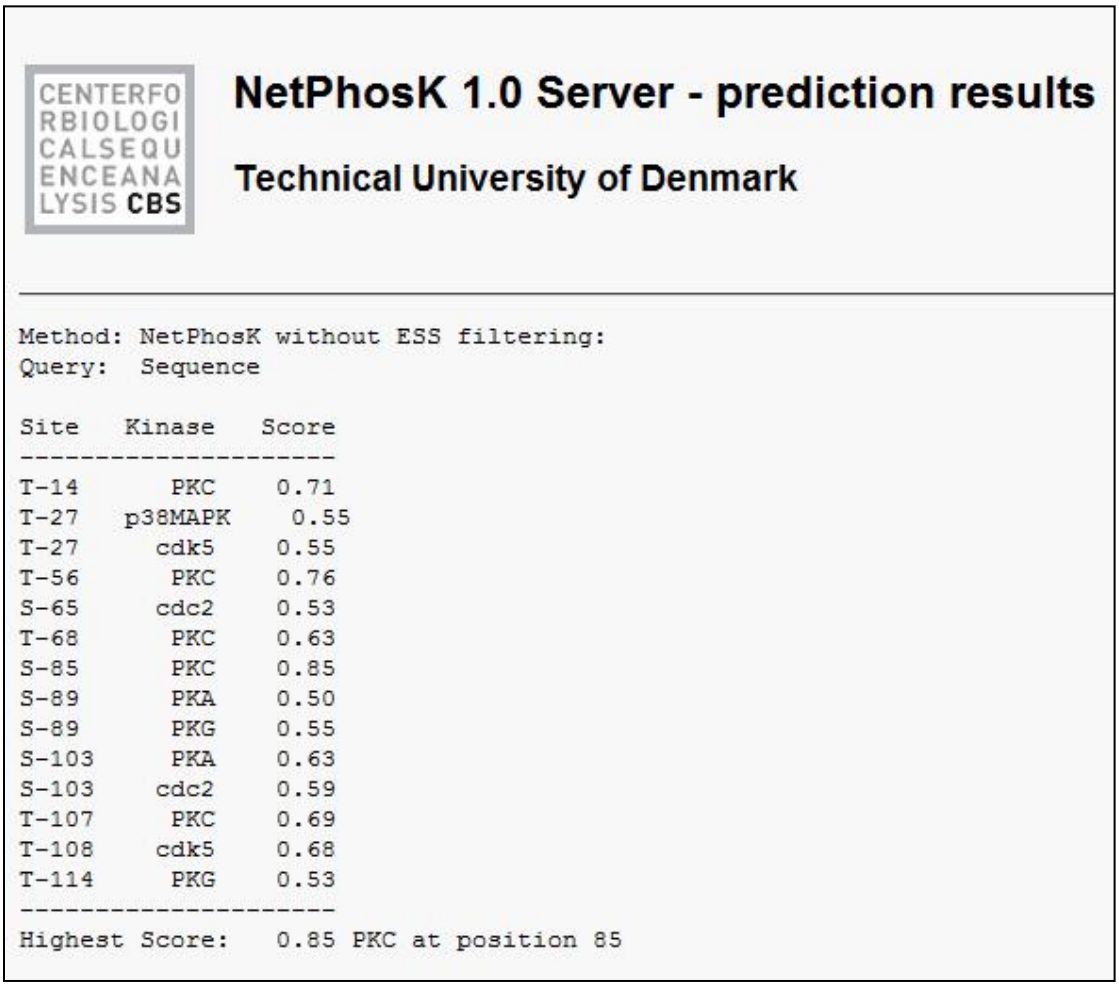
### 3.2.4 Further investigation of the Apoptin specific kinase

Collaborative studies using a microarray method identified potential Apoptin candidate kinases in Multiple Myeloma (MM1) cell lines (Jiang et al., 2010b). A pair of Multiple Myeloma cell lines derived from the same cancer has been conditioned to be more resistant to Dexamethasone (MM1R) with the other being more sensitive (MM1S). These cells have different sensitivity to Apoptin which is inversely proportional to their Dexamethasone sensitivity. Microarray analysis found PKCβ1 was amongst the most differentially expressed kinase in MM1R compared to MM1S by approximately 7-fold. In addition, MERTK (a receptor tyrosine kinase) and DGKH (a kinase involved in the conversion of diacylglycerol into phosphatidic acid) were also found to be overexpressed in MM1R compared to MM1S. This result was then verified by Q-RT-PCR and the microarray data was confirmed (Figure 3.10) with a 6-fold overexpression of PKCβ1 in MM1R. This kinase was further studied, and interaction between Apoptin and PKCβ1 was shown by immunoprecipitation studies and increased phosphorylation of Apoptin when PKCβ1 was overexpressed (Jiang et al., 2010b). This observation was further reinforced by the kinase prediction assay, described below, which identified a potential phosphorylation site by PKCβ1 (Figure 3.9) in close proximity to Threonine 108.

In order to confirm the relevance of potential target kinases in Apoptin phosphorylation or to identify other potential candidates, a kinase prediction assay (Figure 3.9) was performed using the Apoptin protein sequence that looks at phosphorylation sites of a protein and predicts potential kinases that may have target motifs in the sequence.

To confirm these findings in different cell lines and to investigate other potential kinases, a HCT116 group of 3 knockout cell lines were studied. The HCT116 cells used for this study have knocked down expression of p53 and PUMA, a key mediator of p53-dependent apoptosis. These cell lines have different sensitivities to Apoptin, compared to HCT116 wild type (wt), and so were chosen for the microarray analysis. The differences in sensitivity are shown in Figure 3.11, where it can be seen that HCT116 $p53^{-/-}$ are the most sensitive cells to Apoptin induced cytotoxicity, with the $PUMA^{-/-}$ cell line displaying the most resistance to Apoptin. However, this is not significantly greater than in HCT116 wt. Due to the largest differences in Apoptin cytotoxicity being

observed between the PUMA$^{-/-}$ and the p53$^{-/-}$ cell lines, analysis of the microarray data was based on this pair. Table 1 shows that of all the genes that were differentially expressed between these two cell lines, only 5 kinases were up or down regulated by a magnitude of 2 or above. These candidate kinases were further investigated by analysing the sequence homology and potential phosphorylation motifs for these kinases on the Apoptin sequence (Figure 3.9). The analysis coupled with findings from our collaborative work found that the kinases that were flagged by the microarray were not relevant to Apoptin phosphorylation in this system. By using the microarray data, it was also possible to further analyse the targets knocked down using the siRNA library. Figure 3.12 shows that the kinases that were targeted by the library had little or no difference in expression between the Apoptin resistant and sensitive cell lines, with a maximum change of 2-fold in kinase expression.



**NetPhosK 1.0 Server - prediction results**

**Technical University of Denmark**

```
Method: NetPhosK without ESS filtering:
Query:  Sequence

Site   Kinase   Score
---------------------
T-14      PKC    0.71
T-27   p38MAPK    0.55
T-27     cdk5    0.55
T-56      PKC    0.76
S-65     cdc2    0.53
T-68      PKC    0.63
S-85      PKC    0.85
S-89      PKA    0.50
S-89      PKG    0.55
S-103     PKA    0.63
S-103    cdc2    0.59
T-107     PKC    0.69
T-108    cdk5    0.68
T-114     PKG    0.53
---------------------
Highest Score:   0.85 PKC at position 85
```

**Figure 3.9 Kinase prediction assay data.** Data from the online kinase prediction software detailing kinases with potential phosphorylation motifs in the Apoptin sequence.

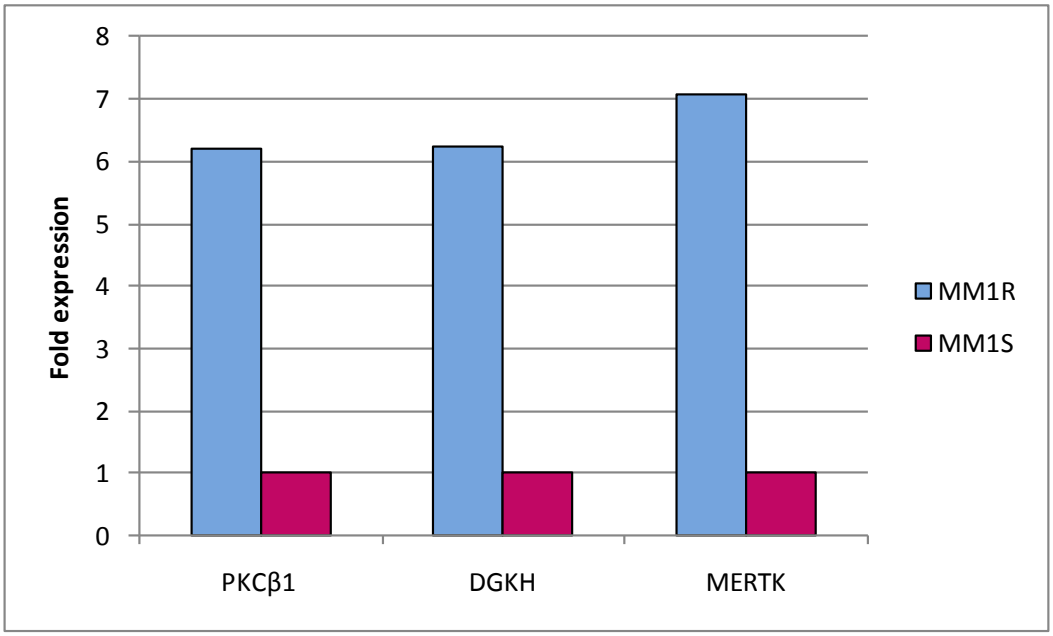



**Figure 3.10 Fold expression of the three most differentially expressed kinases in MM1 cells validated by Q-RT-PCR.** Quantification of Q-RT-PCR data showing the differences in expression of PKCβ1, DGKH and MERTK between MM1R and MM1S cells.

**Table 1 Microarray kinase analysis of HCT116 panel of knockout cell lines**

| Kinases overexpressed in HCT116 PUMA$^{-/-}$ vs p53$^{-/-}$ | Fold overexpression | Kinases overexpressed in HCT116 p53$^{-/-}$ vs PUMA$^{-/-}$ | Fold overexpression |
|---|---|---|---|
| SLK | 2.18 | RIOK1 | 3.36 |
| | | PIK3AP1 | 2.35 |
| | | PRKCA | 2.043 |
| | | ZAK | 2.01 |

**Table 2 Microarray kinase analysis of HCT116 panel of knockout cell lines**

| Kinases overexpressed in HCT116 wt vs p53$^{-/-}$ | Fold overexpression | Kinases overexpressed in HCT116 p53$^{-/-}$ vs wt | Fold overexpression |
|---|---|---|---|
| ARAF | 4.23 | CHPT1 | 3.02 |
| NME1 | 3.27 | CAMK2D | 2.63 |
| SYK | 3.02 | LMTK3 | 2.37 |
| CDC7 | 2.68 | PCK2 | 2.29 |
| TTK | 2.52 | TRIB3 | 2.28 |
| PLK4 | 2.31 | SGK | 2.24 |
| YES1 | 2.11 | DDR1 | 2.24 |
| CHEK1 | 2.03 | RPS6KA2 | 2.23 |
| | | NUAK1 | 2.16 |
| | | C9orf103 | 2.09 |
| | | PINK1 | 2.02 |

**Table 3 Microarray kinase analysis of HCT116 panel of knockout cell lines**

| Kinases overexpressed in HCT116 wt vs PUMA -/- | Fold overexpression | Kinases overexpressed in HCT116 PUMA -/- vs wt | Fold overexpression |
|---|---|---|---|
| SYK | 2.77 | MAP4K1 | 2.47 |
| CTBP1 | 2.60 | PRKCZ | 2.44 |
| CSNK2A1 | 2.38 | PCTK3 | 2.33 |
| DPAGT1 | 2.17 | RPS6KA2 | 2.23 |
| TCEAL8 | 2.10 | CDC2L6 | 2.21 |
| DCK | 2.10 | NME7 | 2.11 |
| ANP32C | 2.08 | ULK1 | 2.07 |
| PRKAR1B | 2.07 | NUAK1 | 2.03 |
| MPHOSPH10 | 2.03 | MAST1 | 2.01 |
| | | GNE | 2.01 |

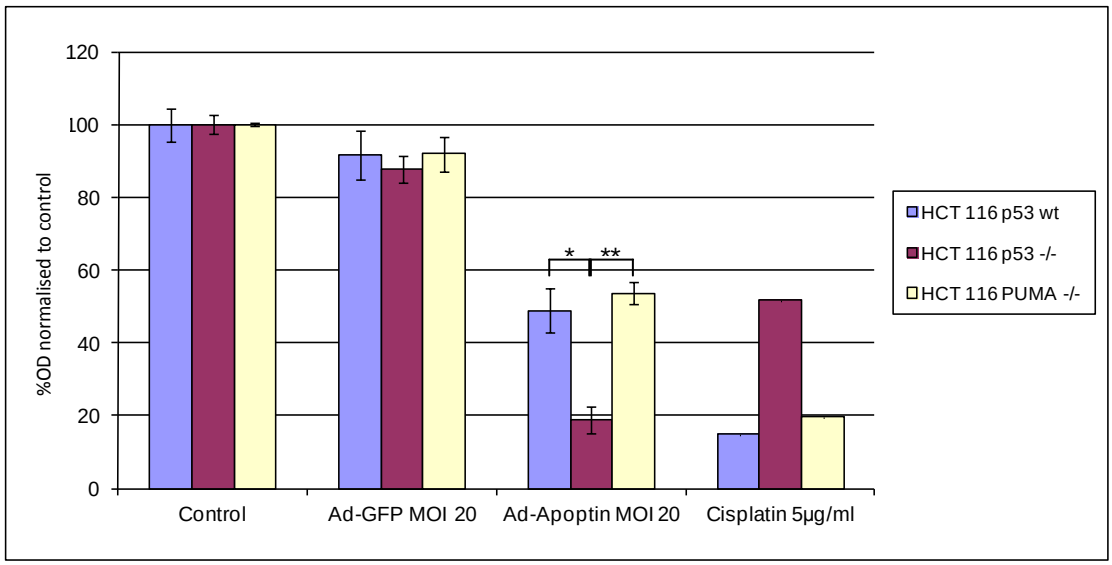



**Figure 3.11 HCT116 panel of knockouts tested for Apoptin sensitivity.** MTT analysis showing differing sensitivity to Apoptin after 4 days of three HCT116 cell lines used for the microarray experiment. Normalised to control values. Error bars indicate stdev. n=3.

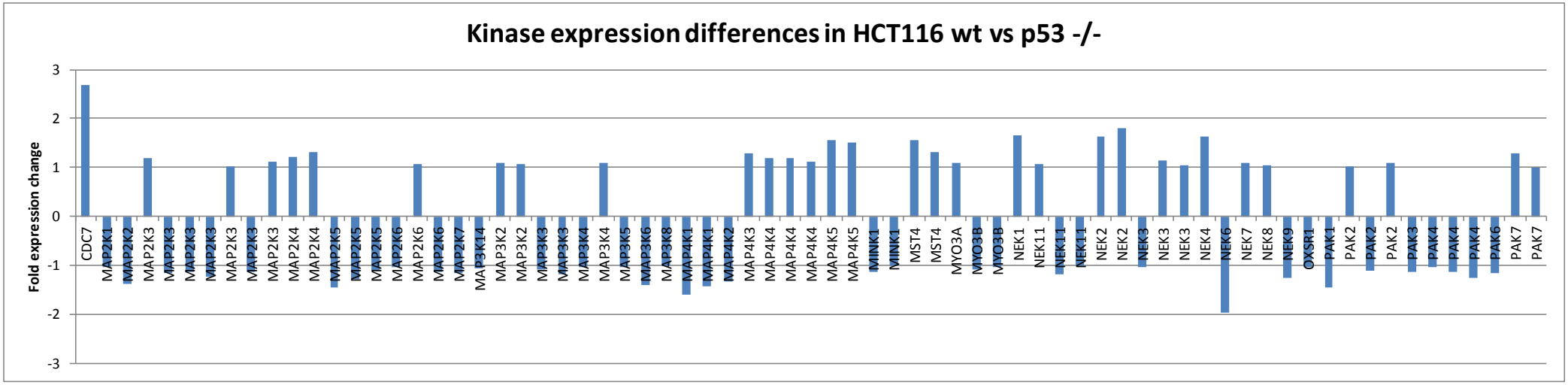



**Figure 3.12 Expression profile of the kinases targeted in the siRNA library.** Quantification of the microarray analysis of only the kinases targeted by the siRNA library showing minimal differences in expression between the Apoptin sensitive and resistant cell lines.

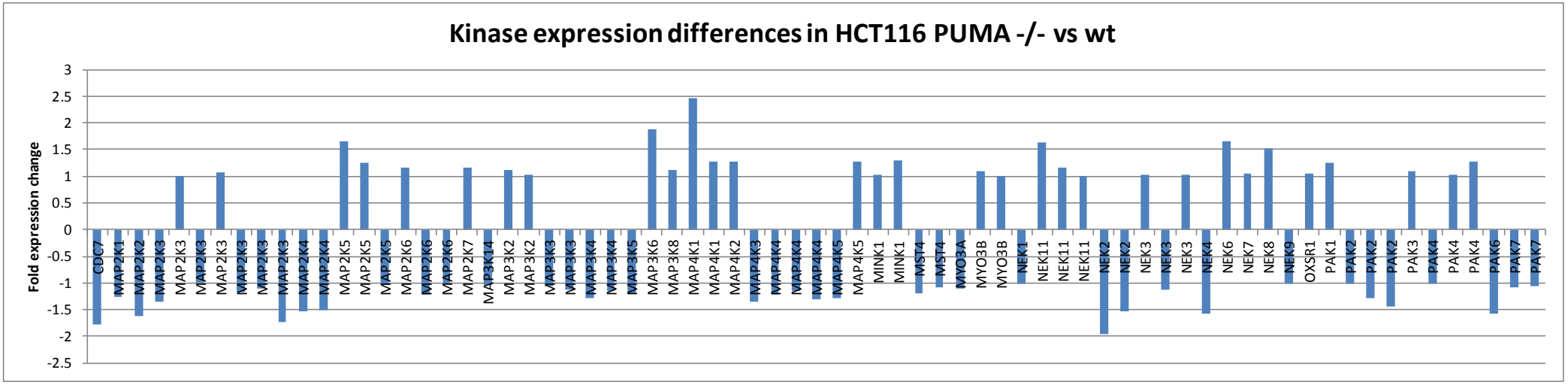



**Figure 3.13 Expression profile of the kinases targeted in the siRNA library.** Quantification of the microarray analysis of only the kinases targeted by the siRNA library showing minimal differences in expression between the Apoptin sensitive and resistant cell lines.

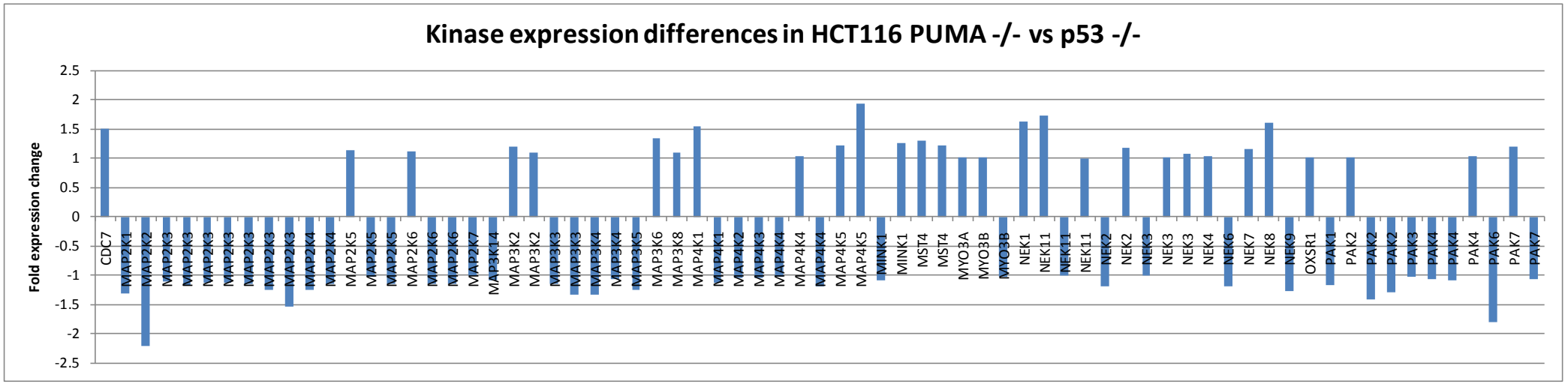



**Figure 3.14 Expression profile of the kinases targeted in the siRNA library.** Quantification of the microarray analysis of only the kinases targeted by the siRNA library showing minimal differences in expression between the Apoptin sensitive and resistant cell lines.

## *3.3 Discussion and Conclusions*

The focus of this work was to determine kinase activity involved in Apoptin cytotoxicity specific to tumour cells using different approaches. Initial studies to determine the effectiveness of the two cell viability assays showed minimal difference in cell death using cisplatin. However, once infection with Apoptin adenoviral vectors was studied, variation in the two viability assays was observed. The decision to use MTT for the future viability assays was due to lack of consistency when using cell titre glo for viral infection. Explanation of the observed variation in measurements could be attributed to the differing methods by which the cell titre glo assay records cellular viability. This assay determines the number of viable cells in culture based on the concentration of ATP, as an indicator of metabolically active cells. MTT also functions by a similar method, however, the result is determined by the actual process of metabolism as opposed to a by product of metabolism.

Further MTT cellular viability assays showed that from a group of candidate cell lines, HCT116 cells were most suitable for the siRNA library screen. Limitations in siRNA technology meant that a window of 72 hours was available once the cells were treated with siRNA before the effects of the interference were lost. The intention of this experiment was to find a cell line that could be infected with Apoptin and show the most significant killing compared to controls in a time point of 48 hours. This would ensure that silencing of the particular genes of interest was still at the maximum possible level during treatment with Apoptin, avoiding false results.

The siRNA library analysis of HCT116 cells infected with Apoptin showed no significant effects of kinase knockdown on Apoptin sensitivity by MTT analysis when compared to controls for Apoptin mediated cell death. Variation in cellular viability is seen amongst the different kinases silenced; however, this variation is attributable to the kinase knockdown itself. This library targets the p38 MAPK signalling pathway family of kinases which has been implicated in a range of responses from apoptosis to cell cycle regulation, differentiation, survival and transformation (Whitmarsh and Davis, 1996). As it covers a wide range of functions within the cell, knockdown of members of this pathway would have effects resulting in observations such as reduced viability, seen in Figure 3.8. The decision to use such a library for the present study originated in

published work on p38 MAPK signalling and its involvement in cancer (Loesch and Chen, 2008; Taylor et al., 2008).

Studies have shown that Apoptin, once in its active form in a transformed cell, initiates cell cycle arrest at the G2/M checkpoint, before inducing apoptosis (Teodoro et al., 2004a). This G2 arrest could be linked to the MAPK signalling pathway as studies have shown that G2 phase arrest requires the phosphorylation of p38 MAPK after gamma radiation (Wang et al., 2000) and exposure to ultraviolet light (Bulavin et al., 2001). Another tumour-specific protein, HAMLET, also induces apoptosis in transformed cells and this cytotoxicity has been linked to the MAPK signalling pathway (Cuadrado et al., 2007). As an initial library for identifying some targets for further study, the MAPK library was considered a good candidate, even though a full human kinome library was available. The library including all known human kinases could have identified the Apoptin kinase or kinases. However, the high throughput nature of such a study would have made a full kinome library open to larger discrepancies and errors. The human kinome library covers over 700 targets, as opposed to the 54 available in this library, and a large number of these would be irrelevant, as was seen here. Further investigation of an Apoptin specific kinase would therefore need to be more specific to allow identification of true targets, for example a more in depth focus on a smaller number of kinases.

In addition to PKC and PKG being flagged in the kinase prediction software (Figure 3.9), a number of other kinases were also predicted to have some association with Apoptin. Specifically CDK5 was found at T108, the residue thought to be important for Apoptin tumour specificity. However, because this kinase is found mostly active in the nervous system and is involved in development of neurons it was dismissed during this study to focus on what appeared to be more relevant targets: PKC and PKG (Cheung and Ip, 2004; Huang et al., 2010; Zhang et al., 2008). Interestingly, when analysing the data for the kinase prediction assay, p38MAPK was flagged as a potential kinase with a consensus motif in the Apoptin sequence. The particular motif was not the T108 residue and, in addition to the siRNA library data, any potential relevance of this kinase and its pathway was ruled out.

The microarray analysis of the Apoptin sensitive and more resistant HCT116 cell lines, failed to uncover any kinases with a significant difference in expression as was found using the multiple myeloma model cell lines (Jiang et al., 2010b). Any

kinases that were flagged had no potential known links to Apoptin in terms of potential phosphorylation motifs and target interaction. PKCα, for example, was shown not to interact with Apoptin in IP studies (Jiang et al., 2010b) whereas PKCβ1 was found to be important for Apoptin phosphorylation, but was not flagged in the microarray analysis. In addition, neither DGKH nor MERTK were flagged in the microarray analysis of HCT116 cells whereas they were significantly differentially expressed along with PKCβ1 in the multiple myeloma studies.

Further to analysing the total kinase expression, the siRNA targets were also investigated for differential expression between the HCT116 cell lines. Figures 3.12, 3.13 and 3.14 show the data for this analysis which highlights the lack of significant change in expression of these kinases between the cell lines, further suggesting that the library was not relevant to this current study.

The results of the microarray in addition to the siRNA library analysis show that there is no significant difference in kinase expression between the two cell lines with respect to their sensitivity to Apoptin. These particular cell lines have knocked down expression of p53 and p53 target, PUMA, which are important in apoptosis. The differences seen in Apoptin sensitivity could more likely be attributed to the abrogated expression of p53 proteins, and the subsequent effects of that modulation. However, it has been shown by many studies (Backendorf et al., 2008a; Schoop et al., 2004; Zhuang et al., 1995b) that Apoptin induced apoptosis is a p53 independent mode of cell death, as cell lines without p53 are shown to be sensitive to Apoptin, as is shown by this study. Cisplatin induced apoptosis, which is shown to be p53 dependent (McCurrach et al., 1997; Vasey et al., 1996), is affected by knockdown of p53 in this study (Figure 3.9) where $p53^{-/-}$ cells are more resistant to cisplatin's effects than the wt or $PUMA^{-/-}$ cells. Because of these findings it was considered that these cell lines would be good candidates for the study of kinase expression related to Apoptin sensitivity.

As an alternative to the microarray and siRNA library, Figure 3.9, the Apoptin kinase prediction assay highlighted a number of potential kinases for investigation, one such kinase was PKG. According to the data presented in Figure 3.9, PKG has potential phosphorylation sites on Apoptin at different residues to the T108 site. Interestingly, studies have suggested an involvement of PKG in the transition from normal to transformed cells. More specifically, PKG expression is shown to be lost once a cell becomes transformed or tumourigenic (Fujii et al., 1995b; Kwon et al.,

2008a). Due to the characteristic expression of PKG in Apoptin resistant cell lines, it was hypothesised that this kinase could have a function regulating Apoptin induced cell death.

In summary, the data presented here describes PKCβ1 and more tentatively, PKG as potential interacting kinases that could affect the cytotoxic ability of Apoptin in different cell lines. The expression of kinases could be overlooked in this particular study as the relevance appears to be minimal. As with many proteins that are shown to have similar transcriptional activity, this does not always equate to similar protein expression levels, and, with regards to kinases, potential activity. In the multiple myeloma model cells; however, PKCβ1 transcriptional activity and protein expression level did correlate with the kinase's potential effect on Apoptin cytotoxicity. However, the difference in PKCβ1 expression between Apoptin sensitive and resistant cells was significantly different. In the HCT116 study, no such kinase was found to be expressed significantly higher in the more sensitive cell lines, indicating that kinase expression levels alone may not be sufficient as a marker for Apoptin sensitivity in this particular model. Further investigation will include PKCβ1 and PKG and their interaction and effect on Apoptin induced cell death.

# Chapter 4

# Characterisation of PKG and PKC in normal and tumour cells in relation to Apoptin sensitivity

## *4.1 Introduction*

Kinases are a group of enzymes that catalyse many reactions involved in cellular signalling by phosphorylating a multitude of targets including structural proteins, signalling molecules and other kinases. Previously, it was explained that Apoptin is phosphorylated on a threonine residue in transformed cells. This prompted the current study to pursue serine/threonine kinases as potential targets for Apoptin phosphorylation. One potential serine/threonine kinase implicated by our work is PKCβ1 (Jiang et al., 2010b).

Studies from our group showed that PKCβ1 (a classical PKC isoform) was implicated in Apoptin induced apoptosis in transformed cell lines, where there was clear evidence of interaction with Apoptin (Jiang et al., 2010b). In order to further investigate the potential significance of PKCβ1 in Apoptin induced apoptosis, cell specific expression and localisation of PKCβ1 was studied with respect to Apoptin sensitivity.

Apoptin phosphorylation motif prediction studies highlighted a further serine/threonine kinase, PKG, as a potential Apoptin kinase. This kinase family is responsible for a wide range of physiological processes including, cell motility, gene expression and apoptosis. A recent study has shown that PKG-I is involved in the wnt signalling pathway and is a potential regulator of β-Catenin signalling (Kwon et al., 2010). In other studies PKG-I expression has been shown to be lost in cells that have become transformed or tumourigenic (Hou et al., 2006), this phenomenon is also observed during the passaging of certain primary cell lines (Lin et al., 2004b). PKG-I is noted for its anti-tumour characteristics and the ability to induce growth arrest or apoptosis in a variety of tumour cell lines (Chan and Fiscus, 2003; Deguchi et al., 2004; Kwon et al., 2008b).

Based on our initial findings of the kinase prediction assay for Apoptin and the studies described above, we decided to further study PKG-I as a potential negative regulator of Apoptin sensitivity. Expression and localisation of PKG-I in tumour and normal cells and the effect on Apoptin sensitivity was investigated.

## *4.2 Results*

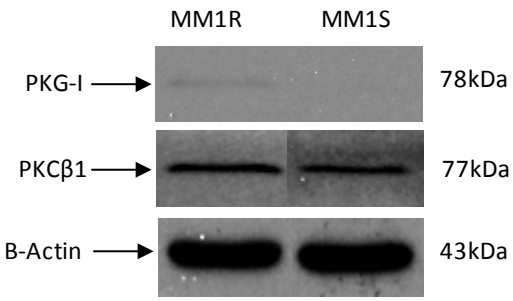
### 4.2 Analysis of expression of PKG-I and PKCβ1 in normal and tumour cell lines

The aim of the following experiments was to determine expression characteristics of a selection of matched pairs of normal and tumour cell lines. This was done using Western blot analysis to investigate the relative levels of PKG-I and PKCβ1 proteins.

### 4.2.1 Expression of PKG-I and PKCβ1 in Multiple Myeloma cell lines

Using the Multiple Myeloma matched pair cell lines described in Chapter 3, expression of PKG-I and PKCβ1 proteins was evaluated. According to Western blot analysis (Figure 4.1), which is confirmed by the Q-RT-PCR in Chapter 3, PKCβ1 is at a higher level in MM1R cells compared to MM1S. PKG-I expression, in contrast, is shown to be detected but only at low levels (Figure 4.1).

**A**




**B**

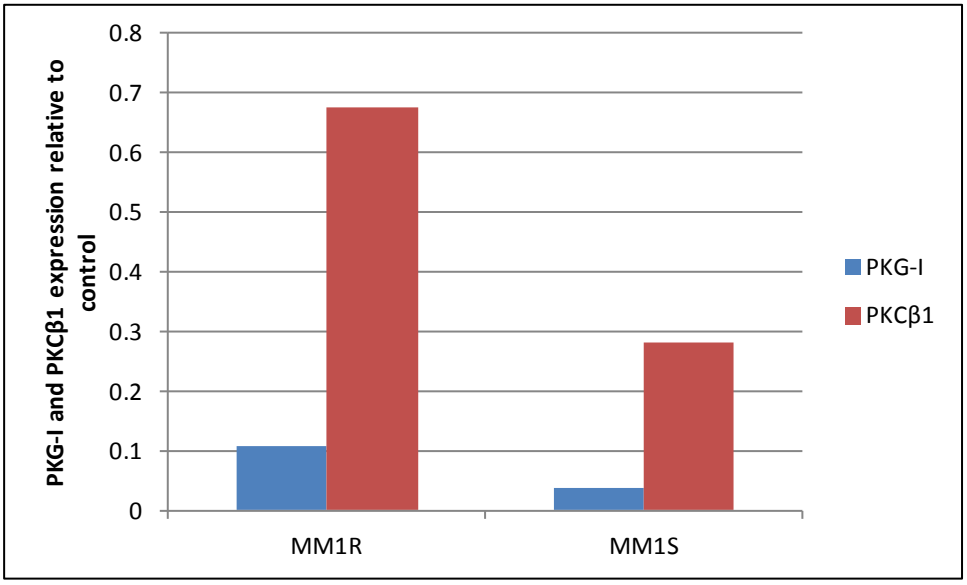



**Figure 4.1 PKG-I and PKCβ1 expression in MM1R and MM1S cell lines.** Determined by Western blot analysis (A) and quantification, using ImageJ software, of expression (B). Cells were grown for 72 hours. Blots in (A) are from the same blot, but were modified to remove unrelated data. Blots were exposed for 1 minute each probe. PKG-I and PKCβ1 were analysed from the same blots where PKG-I was probed first. B-Actin was probed once at the same time as PKG-I. ImageJ quantification was performed by measuring the area and density of each band and comparing them with the B-Actin control.

### 4.2.2 Expression of PKG-I and PKCβ1 in Head and Neck cancer cell lines

Head and Neck squamous cell carcinoma (HNSCC) cell lines were also used to investigate differences in the expression of PKG-I and PKCβ1 proteins. Four pairs of primary and metastatic HNSCC cell lines were used to analyse changes in kinase expression in relation to metastatic status. Western blot analysis found that in general, the more metastatic of each paired cell line expressed higher levels of PKCβ1, however, the relative expression of PKG-I was similar between the pairs (Figure 4.2).

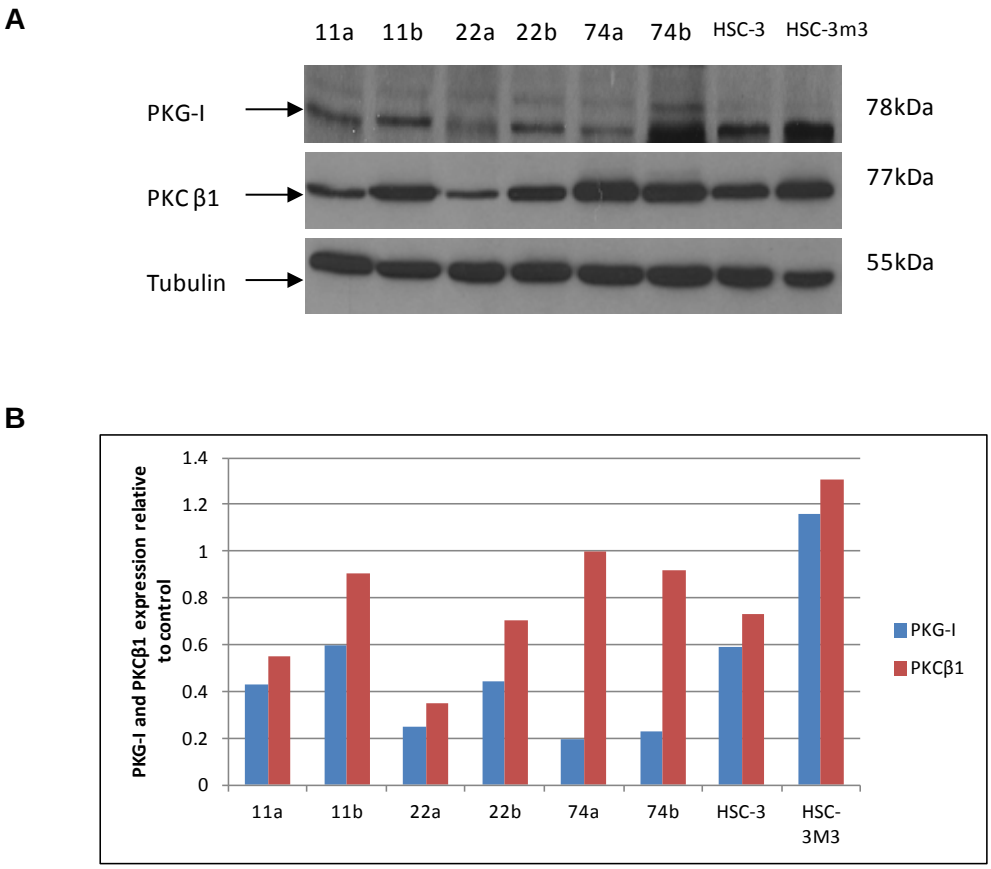



**Figure 4.2 PKG-I and PKCβ1 expression in Head and Neck cancer cell lines.** Determined by Western blot analysis (A) and quantification, using ImageJ software, of expression (B). Pairs of parental and their more metastatic tumour cell lines were used, where a = parental and b = metastatic. HSC-3 is parental and HSC-3M3 is metastatic. Cells were grown for 4 days.

### 4.2.3 Expression of PKG-I and PKCβ1 in Colon cell lines

HCT116 colon carcinoma and NCM460 normal colon cell lines were studied for expression of PKG-I and PKCβ1 proteins in relation to their transformed and normal cell status. Western blot analysis showed that the HCT116 tumour cell line had increased levels of PKCβ1 protein when compared to the normal NCM460 cells (Figure 4.3) whilst PKG-I expression seemed to be higher in NCM460 cells compared to HCT116 cells (Figure 4.3).

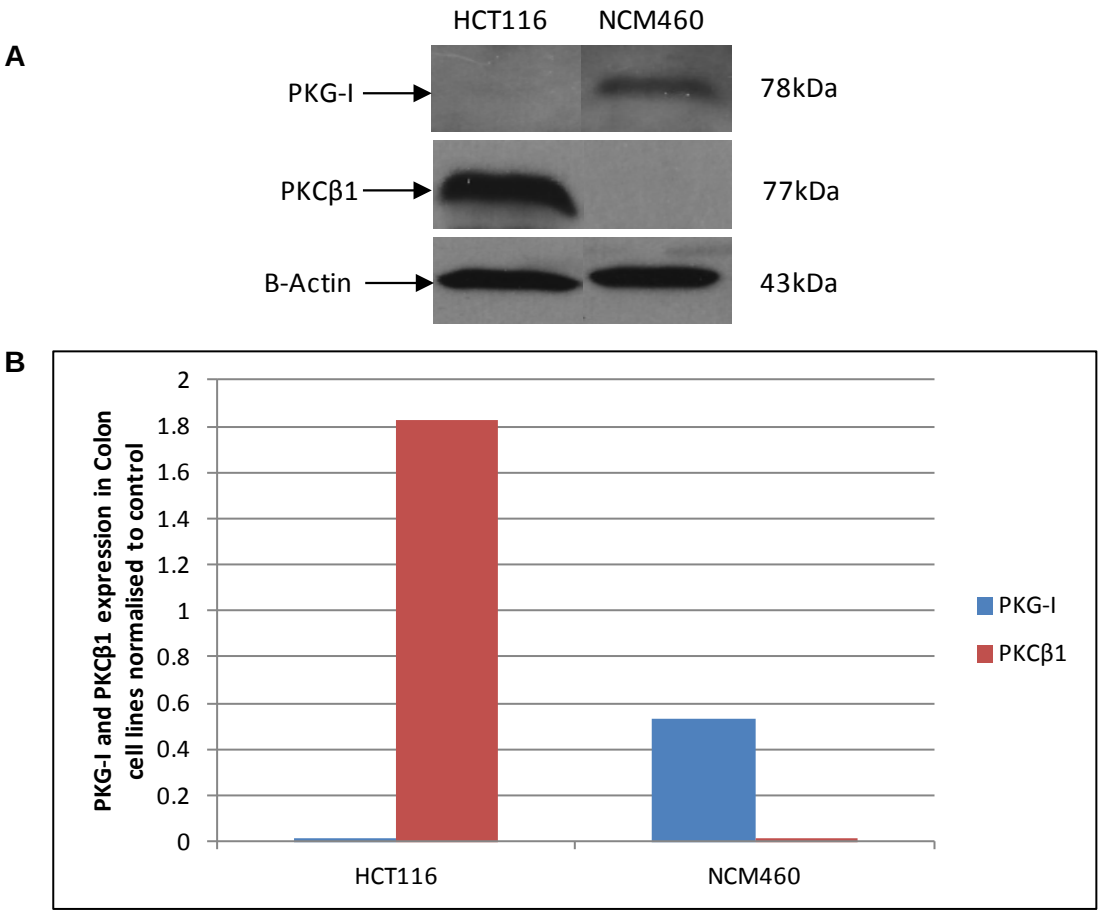



**Figure 4.3 PKG-I and PKCβ1 expression in HCT116 tumour cells and NCM460 normal cells.** Determined by Western blot analysis (A) and quantification, using ImageJ software, of expression (B). Cells were grown for 72 hours. The figure in panel A is constructed from the same blot, but modified to remove unrelated data. Blots were exposed for 1 minute each probe. PKG-I and PKCβ1 were analysed from the same blots where PKG-I was probed first. B-Actin was probed once at the same time as PKG-I. ImageJ quantification was performed by measuring the area and density of each band and comparing them with the B-Actin control.

### 4.2.4 Expression of PKG-I and PKCβ1 in Colon cell lines

HCT116 cell lines with loss of p53 or PUMA expression were studied for their sensitivity to Apoptin induced cell death, as described in Chapter 3. In order to further examine the relationship between the sensitivity of these cells and the expression of PKG-I and PKCβ1, Western blot analysis was used (Figure 4.4). The results in Figure 4.4 show that PKCβ1 is expressed at a higher level in all cell types in this panel compared to PKG-I, which has an expression level below the level of detection. However, the variation in the expression of PKCβ1 does not appear to correlate with Apoptin sensitivity in this panel (Figure 3.11).

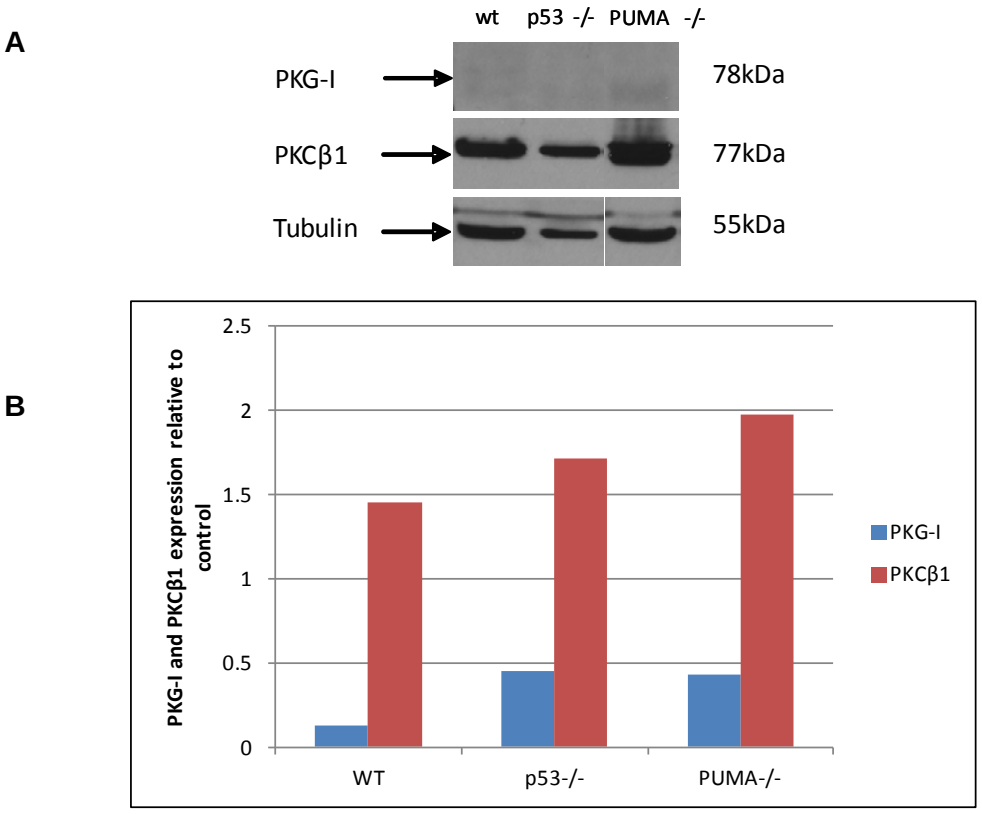



**Figure 4.4 PKG-I and PKCβ1 expression in HCT116 wild type and knockout for p53 and PUMA cell lines.** Determined by Western blot analysis (A) and quantification, using ImageJ, of expression (B). Cells were grown for 4 days. The figure in panel A is constructed from the same blot, but modified to remove unrelated data. Blots were exposed for 1 minute each probe. PKG-I and PKCβ1 were analysed from the same blots where PKG-I was probed first. B-Actin was probed once at the same time as PKG-I. ImageJ quantification was performed by measuring the area and density of each band and comparing them with the B-Actin control.

### 4.2.5 Expression of PKG-I and PKCβ1 in Primary Fibroblast cell lines

1BR3 primary fibroblast cell line and its isogenic match the SV40 Large T transformed 1BR3 cell line (1BR3LT) were analysed for PKG-I and PKCβ1 protein expression using Western blot. PKCβ1 was more highly expressed in 1BR3LT compared to 1BR3 in contrast with PKG-I which showed reduced expression in 1BR3LT when compared to 1BR3 (Figure 4.5).

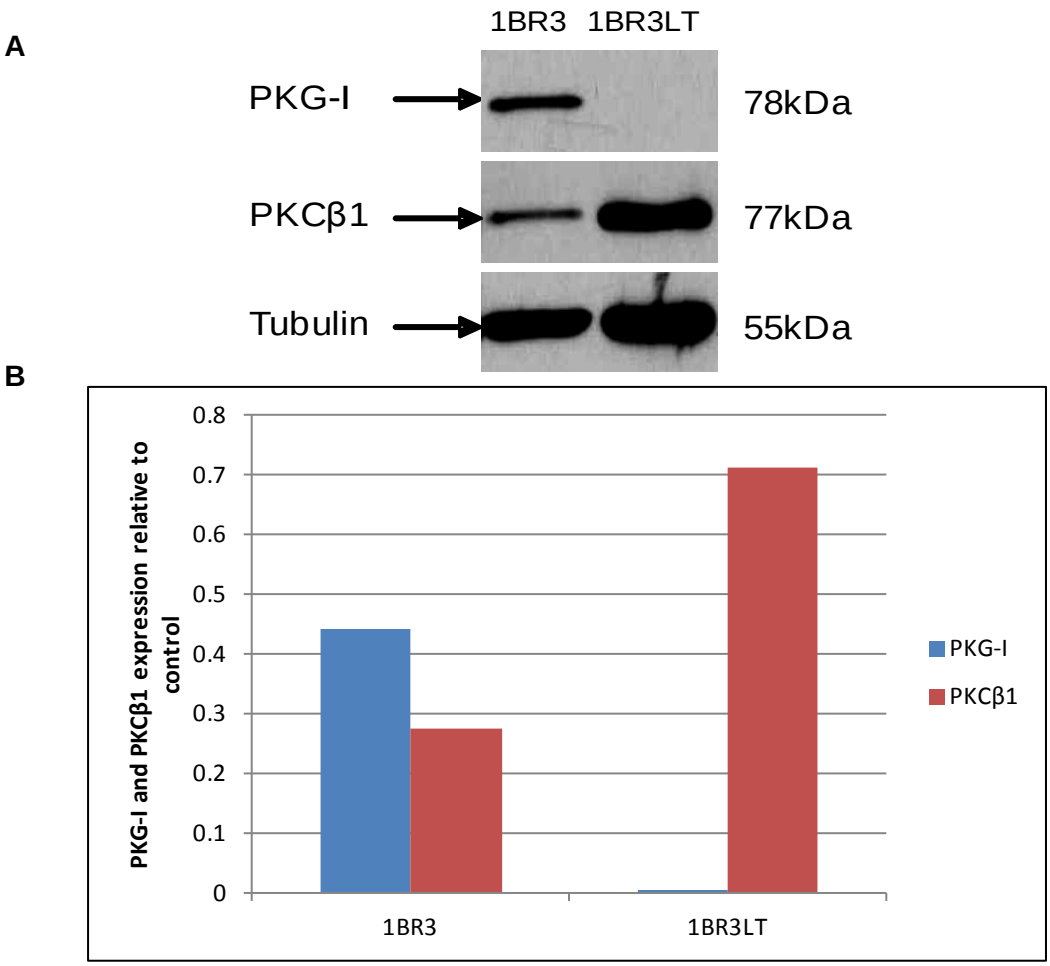



**Figure 4.5 PKG-I and PKCβ1 expression in normal and transformed fibroblasts.** Determined by Western blot analysis (A) and quantification, using ImageJ software, of expression (B). Cells were grown for 72 hours. Blots were exposed for 1 minute each probe. PKG-I and PKCβ1 were analysed from the same blots where PKG-I was probed first. B-Actin was probed once at the same time as PKG-I. ImageJ quantification was performed by measuring the area and density of each band and comparing them with the B-Actin control.

## 4.3 Relative Apoptin sensitivity and relation to expression of PKG-I and PKCβ1

Using a combination of FACS, Western blot and MTT assays, Apoptin sensitivity of the paired cell lines was studied and compared with their expression of PKG-I and PKCβ1. The objective was to determine whether there is any correlation between the expression of these kinases and Apoptin sensitivity in the cell lines tested.

The first cell lines tested were the multiple myeloma pair, the MM1R and MM1S. The relevance of PKCβ1 in Apoptin sensitivity in this model was previously confirmed using Q-RT-PCR (see Figure 3.10). Using Western blot analysis (Figure 4.6) showed higher PARP cleavage in MM1R compared with MM1S 72 hours after infection with a Lentiviral vector expressing Apoptin. This indicated a marked increase in apoptosis in MM1R, suggesting greater sensitivity to Apoptin compared to MM1S. Interestingly, there was a lower level of Apoptin expression in MM1S cells, possibly suggesting increased degradation of Apoptin in this less sensitive cell line. The levels of GFP expression were similar in the two cell lines. This result coincides with increased expression of PKCβ1 in this cell line, shown in Figure 4.1. PKG-I expression in either cell line was found to be very low (Figure 4.1) and did not appear to correlate with sensitivity to Apoptin in this model.

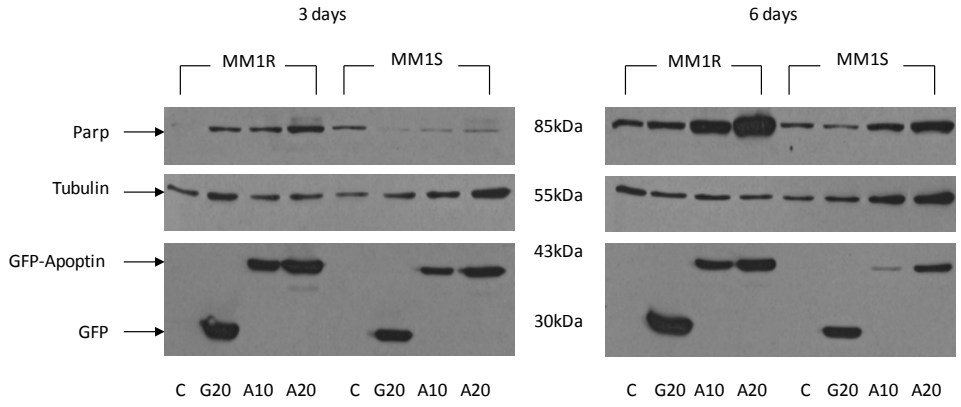
The Head and Neck cancer cell lines showed a variation in Apoptin sensitivity between the pairs of cell lines relative to their status as metastatic. Figure 4.7A shows Apoptin sensitivity of UMSCC 11a and 11b cells using MTT assay, the metastatic line 11b was shown to be more sensitive to Apoptin at both MOIs used; however, the difference is not statistically significant. The 22a and 22b cell lines (Figure 4.7B) show that at an MOI of 30, the metastatic line 22b is more sensitive to Apoptin induced cytotoxicity, but at a higher MOI of 40, 22a is shown to be more sensitive. Figure 4.7C shows the MTT results for the 74a and 74b cell line pair, these cells show a similar response to Apoptin treatment with 74a being slightly more sensitive at MOI 40. These results again were not statistically significant. HSC-3 and HSC-3m3 cell lines were found to have a more marked difference in Apoptin sensitivity at MOI 40, with HSC-3m3 showing approximately 15% more killing in Figure 4.7D. Combining this data (Figure 4.7) with the expression profile (Figure 4.2), it can be seen that in the HNSCC

panel, there is no apparent direct association between PKG-I and PKCβ1 expression and Apoptin sensitivity.

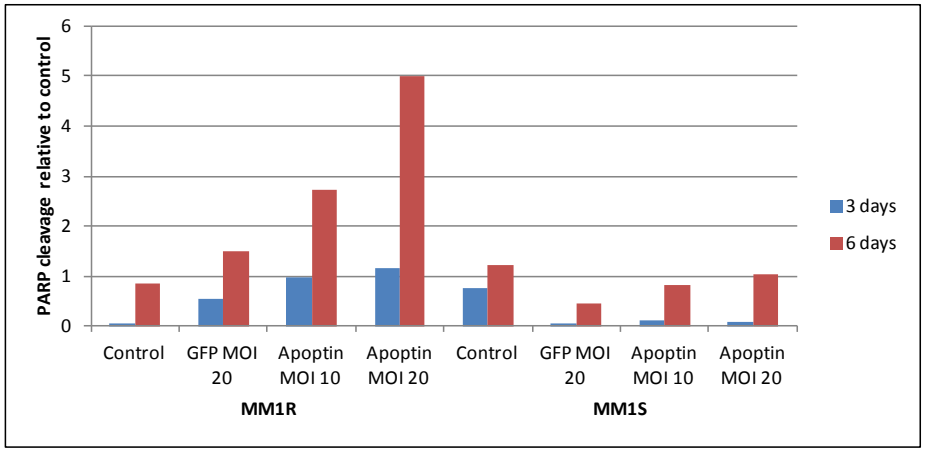
Colon cell lines HCT116 and NCM460 were tested for Apoptin sensitivity using FACS analysis (Figure 4.8) and were compared to the expression of PKG-I and PKCβ1 (Figure 4.3). The results in Figure 4.8 show that HCT116 are 20% more sensitive to Apoptin induced cell death compared to the normal NCM460 cell line. Figure 4.3 shows that HCT116 has a much higher level of PKCβ1 expression than NCM460, with negligible expression of PKG-I. In contrast, NCM460 express PKG-I, but have undetectable levels of PKCβ1, suggesting a link between PKG-I and PKCβ1 expression and Apoptin sensitivity, however, HCT116 were also found to be more sensitive to Cisplatin, another apoptosis inducing agent.

Using a normal fibroblast cell line, 1BR3, and its isogenic SV40 Large-T antigen transformed clone, 1BR3LT, an interesting correlation was observed between the expression of the PKG-I and PKCβ1 kinases and the sensitivity of the cell lines to Apoptin induced cell death. A similar relationship was observed to that found in the colon cell pair, HCT116 and NCM460, where the 1BR3 normal fibroblast cell line had a more pronounced expression of PKG-I compared to the 1BR3LT cell line which had undetectable levels of the kinase (Figure 4.5). A similar relationship was also seen with the expression of PKCβ1, in the transformed cell line 1BR3LT, PKCβ1 expression was significantly higher compared to 1BR3. However, 1BR3 do have detectable levels of PKCβ1 expression. Figure 4.9 shows the FACS analysis of Apoptin sensitivity between 1BR3 and 1BR3LT. The data show 1BR3 cells to be more resistant to Apoptin induced cell death when compared to 1BR3LT, but only with less than 10% difference in killing. A difference in Cisplatin sensitivity is seen in Figure 4.9, as was observed in the colon cell model; however, the difference in the fibroblast pair is more marked.

**A**




**B**




**C**

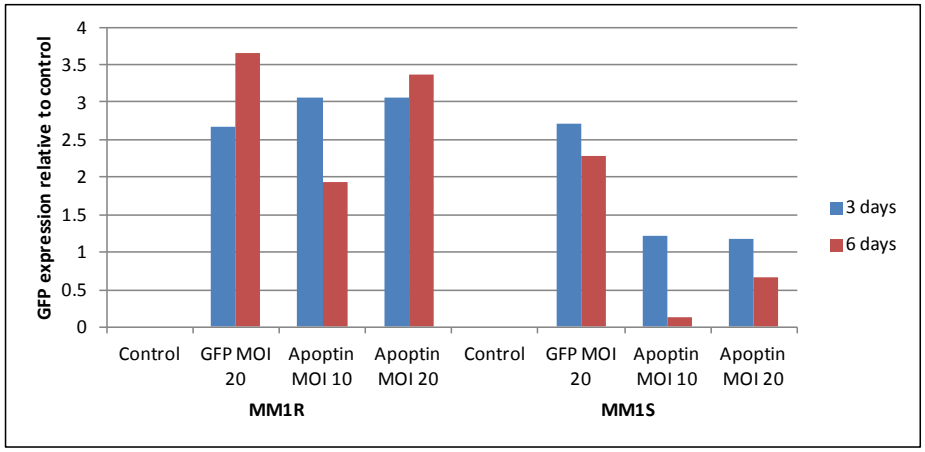



**Figure 4.6 Western blot analysis of Apoptin sensitivity in Multiple Myeloma cell lines determined by PARP cleavage.** Western blot data (A), quantification of PARP cleavage (B) and quantification of GFP expression (C). C = Control, G20 = Adenoviral GFP MOI 20, A10 and A20 = Adenoviral Apoptin MOI 10 and 20, respectively. Cells were grown for 3 and 6 days as indicated.

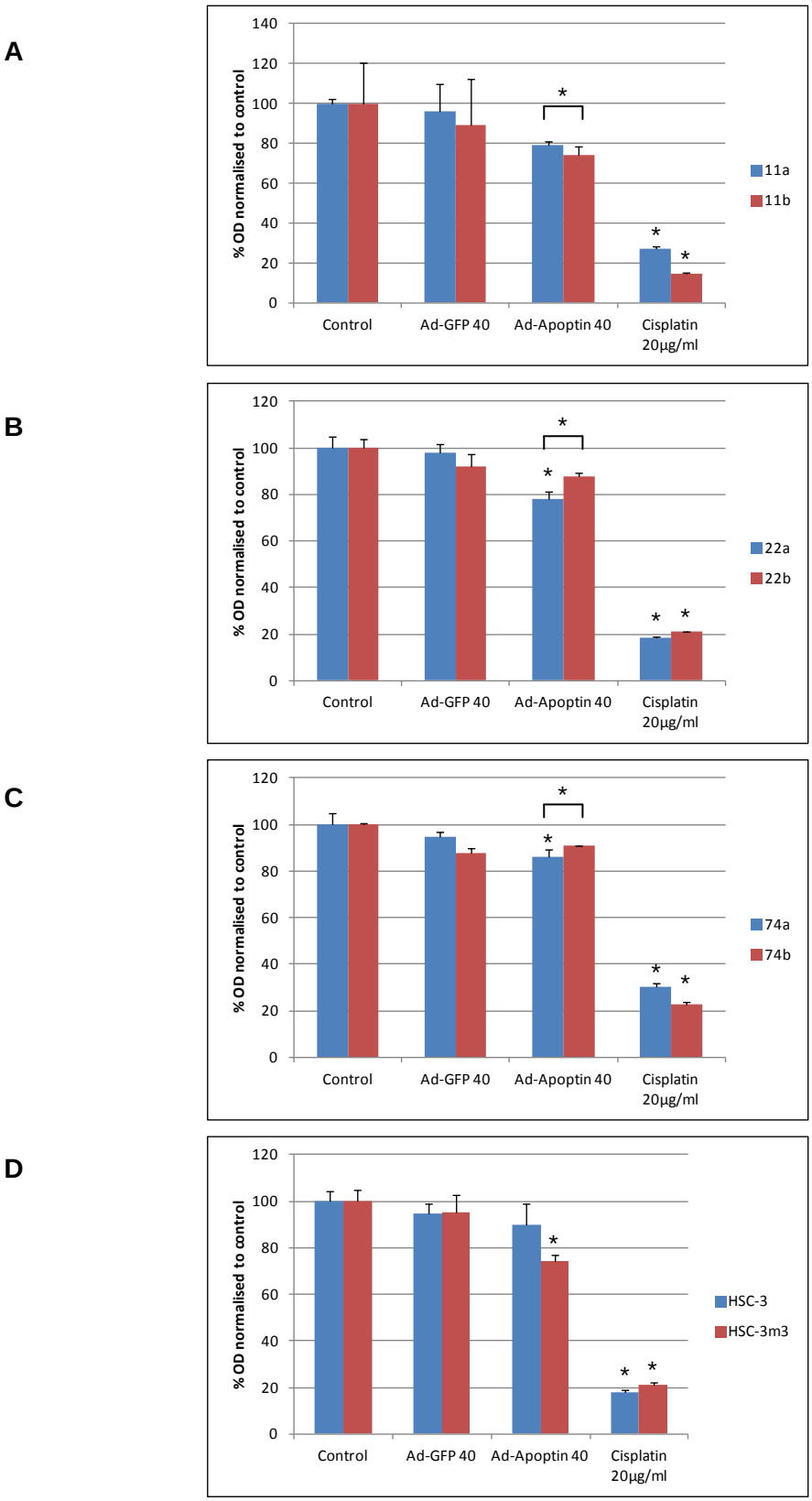



**Figure 4.7 MTT based analysis of Head and Neck cancer panel of cell lines treated with Adenoviral delivered Apoptin.** 11a and 11b (A), 22a and 22b (B), 74a and 74b (C) and HSC-3 and HSC-3m3 (D). Cells were grown for 4 days. Error bars indicate standard deviation. n=3. Cell death shown here is from the total population of cells treated; this data does not take into account efficiency of adenoviral infection.

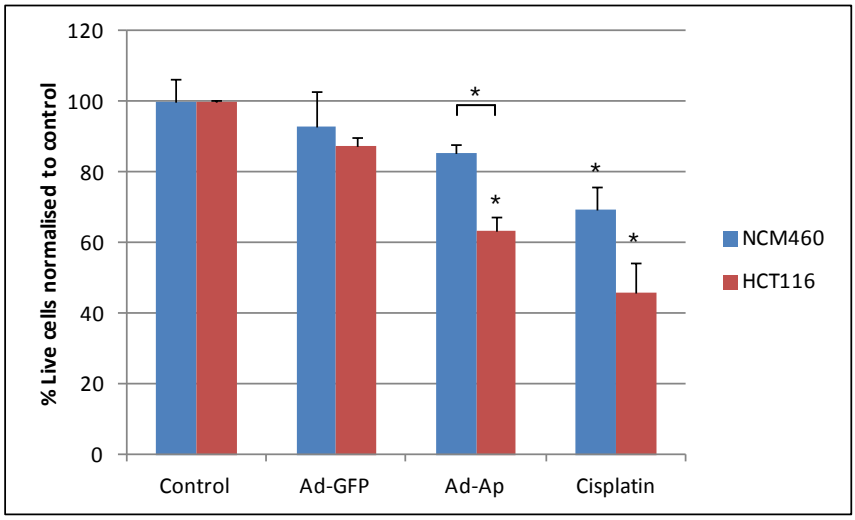



**Figure 4.8 FACS analysis of colon cell lines HCT116 and NCM460 and their sensitivity to Apoptin.** Delivered by Adenovirus at MOI 40. Cells were treated for 72 hours. Error bars indicate standard deviation. n=3. Cell death shown here is from the total population of cells treated; this data does not take into account efficiency of adenoviral infection. For example, cells were not gated for Apoptin or GFP positive expression.

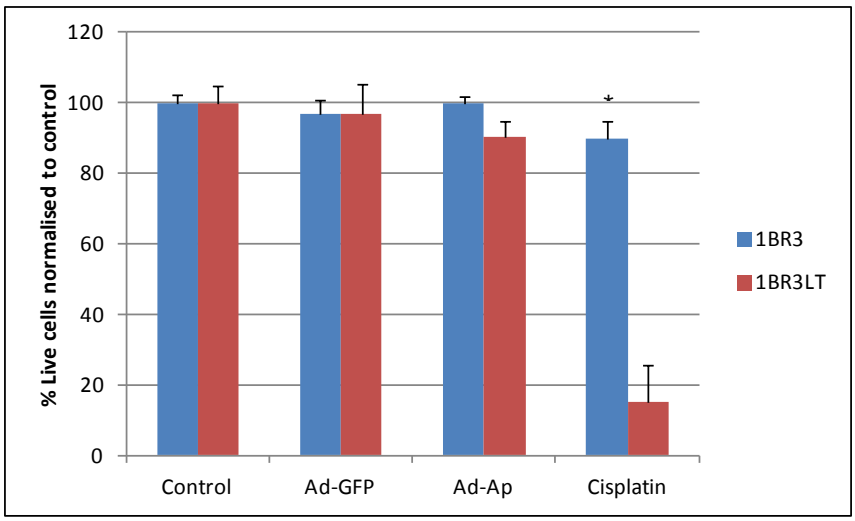



**Figure 4.9 FACS analysis of fibroblast cell lines 1BR3 and 1BR3LT and their sensitivity to Apoptin.** Delivered by Adenovirus at MOI 40. Cells were treated for 72 hours. Error bars indicate standard deviation. n=2. Cell death shown here is from the total population of cells treated; this data does not take into account efficiency of adenoviral infection. For example, cells were not gated for Apoptin or GFP positive expression.

## 4.4 Protein-protein interaction between Apoptin, PKG-I and PKCβ1

Previous results suggested a potential link between PKG-I and PKCβ1 in sensitivity to Apoptin of certain cell lines. This link was further investigated by looking at a physical interaction between Apoptin and PKG-I or PKCβ1.

To investigate protein-protein interaction, HEK293 cells were used due to their ease of transfection and apparent resistance to Apoptin induced cell death. These characteristics provided a suitable model to test ectopic expression of the kinases of interest and Apoptin and any potential interactions. Cells were transfected with differing combinations of plasmids encoding Flag-tagged Apoptin, PKG-I and PKCβ1, incubated for 72 hours and then collected for Immunoprecipitation (IP) analysis as described in Chapter 2.

The results shown in Figure 4.10 indicated physical interaction between Apoptin and PKG-I and between Apoptin and PKCβ1. Figure 4.10A shows that PKG-I and Apoptin are physically bound as pulling down with Flag antibody also pulls down PKG-I (Figure 4.10A), suggesting a physical interaction between this kinase and Apoptin. Although the lane transfected only with PKG-I (Figure 4.10A) also displays a very faint band, this is believed to be due to background because the total protein in Figure 4.10B for PKG-I is similar to the co-transfected band (Figure 4.10B). The interaction between PKCβ1 and Apoptin was also investigated in Figure 4.10C and D. The findings suggest that PKCβ1 also binds to Apoptin (Figure 4.10C). Furthermore, PKCβ1 and PKG-I appeared to physically interact with each other (Figure 4.10C). Collectively, the results suggest that both PKCβ1 and PKG-I physically bind to Apoptin. This result suggests that Apoptin, PKG-I and PKCβ1 may form a complex in the cell and physically interact with one another. This result is interesting as it provides a tentative link between these two kinases and Apoptin.

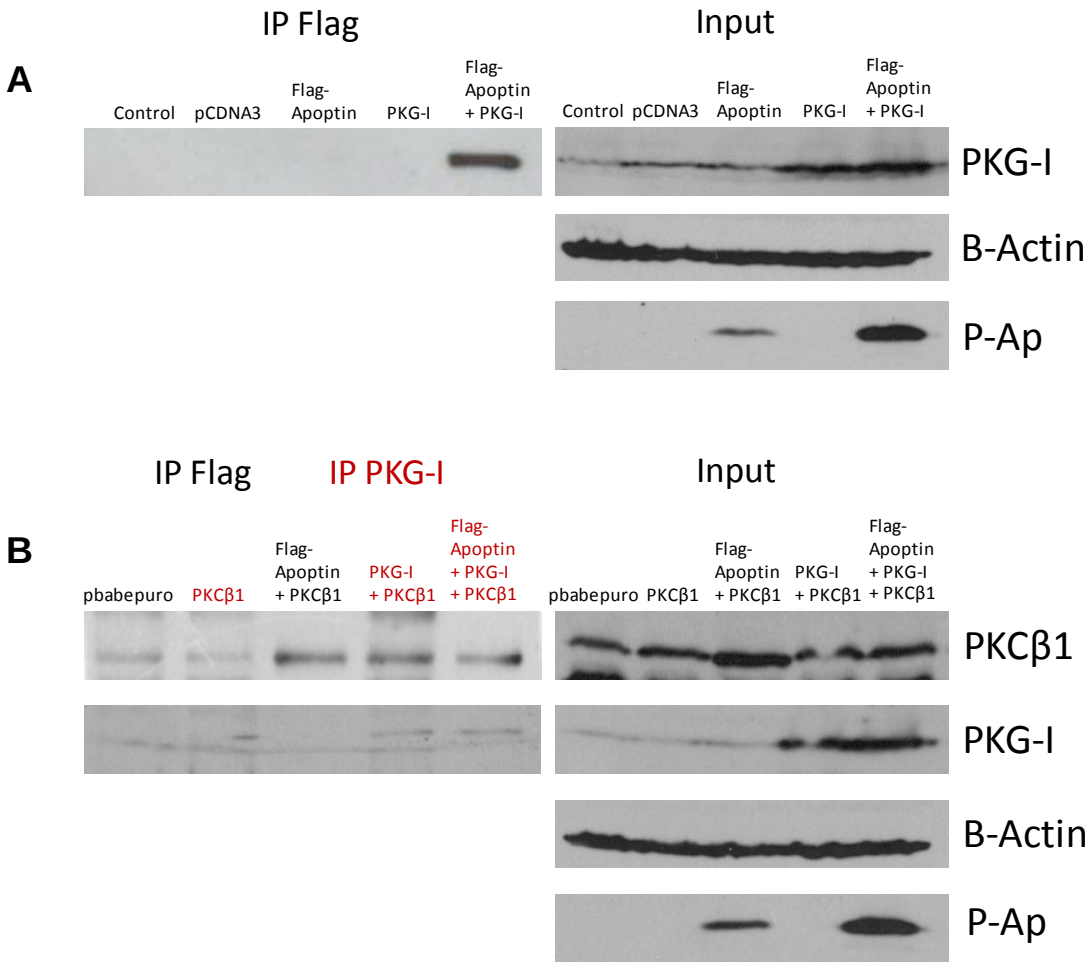



**Figure 4.10 Immunoprecipitation studies in HEK293 cells transfected with a number of different plasmids.** In A, all lanes were immunoprecipitated with Flag antibody. In B, PKCβ1, PKG-I + PKCβ1 and Flag-Apoptin + PKG-I + PKCβ1 (red) were immunoprecipitated with PKG-I antibody, pbabepuro and Flag-Apoptin + PKCβ1 were immunoprecipitated with Flag antibody. Cells were incubated for 72 hours to be consistent with previous experiments. HEK293 are not sensitive to Apoptin induced cytotoxicity. Blots were exposed for 1 minute each probe. PKG-I and PKCβ1 were analysed from the same blots where PKG-I was probed first. B-Actin and P-Apoptin were probed once at the same time as PKG-I.

## *4.5 Discussion and Conclusions*

Apoptin expression induces cell death in transformed, but not normal cells. A number of different processes occur in transformed cells compared to normal cells and these differences need to be studied in order to further understand the function of Apoptin. For example, Apoptin in tumour cells is localised to the nucleus, but in normal cells this nuclear localisation is much less efficient (Danen-Van Oorschot et al., 2003; Guelen et al., 2004). Other studies have also highlighted the importance of phosphorylation of Apoptin for its nuclear localisation and subsequent cytotoxicity (Noteborn, 2009; Tavassoli et al., 2005).

As was discussed in Chapter 3, a tumour specific kinase or kinases could be responsible for the phosphorylation of Apoptin and its resulting toxicity in transformed cells. Previous work highlighted PKCβ1 and potentially PKG-I as kinases involved in Apoptin cytotoxicity, the focus of the work described here was to further investigate these target kinases.

The MM1R and MM1S pair had been used in our published work (Jiang et al., 2010b) and it was important to understand whether or not the kinase expression hypothesis applied to a proven model of variable Apoptin sensitivity. What we found was that PKCβ1 was expressed at a higher level in MM1R than MM1S, as was shown in Chapter 3. In contrast, PKG-I expression was detected at low levels in both cell lines, however, the Apoptin resistant MM1R cells expressed PKG-I at a higher level than the MM1S cells. This result disagreed with the hypothesis and suggested that in the MM pair PKG-I expression was not relevant to Apoptin sensitivity.

To determine the correlation between the expression of these kinases and Apoptin sensitivity, the cells were infected with Lentiviral vectors encoding GFP-Apoptin. Sensitivity was investigated by Western blot using PARP cleavage as an indicator of Apoptosis. The results clearly showed that MM1R cells were more sensitive to Apoptin than MM1S, and this result correlated with a higher expression of PKCβ1. An interesting observation in MM1S cells was that Apoptin expression was reduced compared to MM1R, suggesting that Apoptin was possibly degraded in the less sensitive cell line as GFP expression was similar between the cell lines, the effect of

Apoptin degradation has recently been shown in normal cells in response to Apoptin treatment (Lanz et al., 2012).

The next panel of cell lines investigated was the Head and Neck cancer cell lines. PKG-I was found to be expressed in a similar manner to PKCβ1, with the more metastatic lines showing increased levels of both kinases. The results revealed that although there was variation in sensitivity to cell death in the panel of cell lines, there was no clear trend to link Apoptin sensitivity to PKG-I and PKCβ1 (Table 3); this was also evident in cisplatin sensitivity.

**Table 4 Kinase expression ratio between primary and metastatic cells in HNSCC panel in relation to Apoptin sensitivity**

| | 11a | 11b | 22a | 22b | 74a | 74b | HSC-3 | HSC-3M3 |
|---|---|---|---|---|---|---|---|---|
| PKG-I | 0.42 | **0.58** | 0.36 | **0.64** | 0.45 | **0.55** | 0.34 | **0.66** |
| PKCβ1 | 0.38 | **0.62** | 0.33 | **0.67** | **0.52** | 0.48 | 0.36 | **0.64** |
| % Killing | 20 | **30** | 10 | **15** | **15** | **15** | 10 | **15** |

PKG-I expression has been shown to be lost in cells as they become transformed or tumourigenic (Hou et al., 2006), using a pair of normal and transformed colon cell lines, expression of PKG-I and the relationship to Apoptin sensitivity was investigated. HCT116 cells expressed higher levels of PKCβ1 protein than NCM460 and had completely undetectable levels of PKG-I protein expression. In contrast, NCM460 expressed PKG-I protein at higher levels than HCT116, but no detectable expression of PKCβ1. Cell death analysis shows that HCT116 are more sensitive to Apoptin induced cell death than NCM460. This result supports the hypothesis that PKCβ1 expression is linked to Apoptin sensitivity and PKG-I expression is linked to Apoptin resistance in a colon cancer model.

A HCT116 panel of cell lines including wild type, p53 and PUMA knockout were tested for their sensitivity to Apoptin (Chapter 3). This panel was also tested for kinase expression to investigate the differences in Apoptin sensitivity in relation to PKCβ1 and

PKG-I protein levels. The results found that these cell lines had increasing levels of PKCβ1 expression from wild type to the PUMA$^{-/-}$ cell line. While there was variation in Apoptin sensitivity, all cell lines were sensitive to Apoptin and the expression of PKCβ1 did not appear to correlate with this sensitivity. PKG-I expression was very low in this panel, perhaps contributing to the Apoptin sensitivity of these cells. The difference in sensitivity is shown to be independent of kinase expression, and could be attributable to the p53 and PUMA status of these cell lines.

The primary fibroblast cell line 1BR3, and its SV40 Large T transformed clone 1BR3LT, were used as another model of normal and transformed cell lines to test Apoptin sensitivity and its relationship with kinase expression. As was seen in the colon model, kinase expression was similar; the normal cell line had increased expression of PKG-I and the transformed cell line had increased expression of PKCβ1. Apoptin sensitivity was investigated by FACS analysis similar to the colon model and although the difference in cell death was not as great as in the colon model, it was observed that the 1BR3LT cells were slightly more sensitive to Apoptin induced cell death than 1BR3. The observed low levels of killing in the SV40 transformed 1BR3 cell line when exposed to Apoptin suggest that this model is not very effective in studying Apoptin sensitivity. Cisplatin, however, was again found to have different killing ability between normal and transformed cells, which could suggest that PKCβ1 and PKG-I expression are involved in cell death as opposed to Apoptin induced cell death in particular. Further investigation is required to distinguish between these alternative interpretations. In addition, it is important to note that all FACS data is based on total populations and does not account for low virus infectability. For example, the reason for the low toxicity seen in these cells could be lower viral infection efficiency rather than resistance to Apoptin as shown in Figure 4.11. Cells that are expressing Apoptin are exhibiting early signs of apoptosis; however, these cells are only a small percentage of the total population.

Results show that Apoptin and PKG-I physically bind to one another, evidence of interaction within the cell. In addition, PKCβ1 is shown to bind to Apoptin, which also indicates an interaction between the protein and this kinase. However, the interactions of the other proteins analysed are less convincing. The results indicate that both PKCβ1 and PKG-I bind to each other and also form a complex with Apoptin when all are present within the cell, but the existence of bands in the control lanes of each IP experiment casts doubt on the potential significance of this finding. These could

perhaps be dismissed as background, but further investigation would be required to prove the presented hypothesis, for example, repeat experiments using different complimentary antibodies. These results suggest that there could be a potential physical link between Apoptin and PKCβ1 and PKG-I kinases, however this link is tentative at best. Direct physical interaction does not exclusively determine functional activity and so the ambiguity of these results does not necessarily negate the involvement of PKCβ1 and PKG-I in Apoptin cytotoxic function. Further, the results here describe interactions of exogenously overexpressed kinases with Apoptin; a further examination would benefit from the investigation of endogenously expressed PKCβ1 and PKG-I in a model that more closely resembles cancer.

In summary, the data presented here describes a potential link between Apoptin sensitivity in colon and, less convincingly, fibroblast cell lines and the expression of PKCβ1 and PKG-I. Other cell lines such as MM1 and Head and Neck cancer were found to express these kinases in varying amounts, however, in the case of Head and Neck cancer cell lines the expression did not appear to correlate with Apoptin sensitivity. HCT116 and NCM460 were found to have varying sensitivity to Apoptin that correlated with their expression of PKCβ1 and PKG-I, this was seen to a lesser extent in 1BR3 and 1BR3LT, the fibroblast cell lines.

In order to further this study, the interaction between Apoptin and the target kinases will be tested further by modifying PKCβ1 and PKG-I expression and investigating the effect on Apoptin phosphorylation and cytotoxicity.

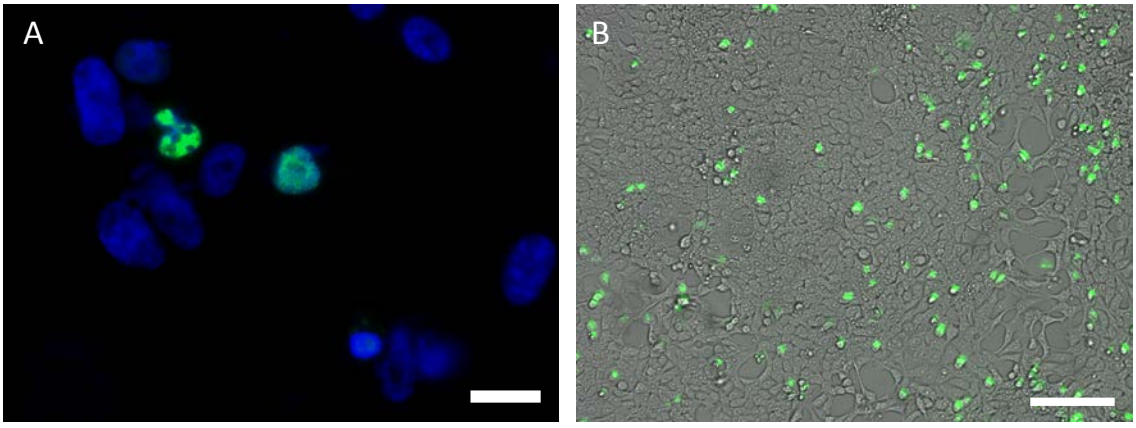



**Figure 4.11 Fluorescence imaging examples of Apoptin infection efficiency and resulting apoptotic effect in 1BR3LT and HCT116.** 60x magnification (Scale bar: 25μm) fluorescence image (A) of 1BR3LT cells infected with Lenti-GFP-Apoptin and stained with DAPI for imaging nuclei. Apoptin infected cells are visible with changed nuclear morphology associated with apoptosis. 10 x magnification (Scale bar: 125μm) fluorescence and brightfield image (B) of HCT116 cells infected with Lenti-GFP-Apoptin showing the infection efficiency generally seen. Of note is the morphology of the cells expressing Apoptin, they are no longer fully adhered to the plate and are beginning to round up, an early sign of cell death. Cells were grown for 72 days.

# Chapter 5

# Modification of PKG and PKC expression and its effect on Apoptin sensitivity

## *5.1 Introduction*

Apoptin phosphorylation in tumour cells has been shown to be important in its cytotoxic effect (Rohn et al., 2002). However, specific kinase/s responsible for Apoptin phosphorylation have yet to be discovered. Cyclic guanosine monophosphate dependent protein kinase I (PKG-I) and Protein kinase C beta 1 (PKCβ1) have been implicated in Apoptin cytotoxicity in this study (see Chapter 4) and in previous studies from this lab (Jiang et al., 2010a).

Using two pairs of PKG-I inducible cell lines in combination with overexpression and knockdown studies; the effect of PKG-I expression on Apoptin phosphorylation and cytotoxicity was investigated. The objective of the studies described in this chapter was to investigate the effects of these kinases, through their modulation, on Apoptin cytotoxicity and phosphorylation. This was a logical extension of our previous studies described in Chapter 4 which had highlighted the potential relationship between PKCβ1 and PKG-I expression and Apoptin sensitivity.

The aim of this study was to confirm these data by knocking down or overexpressing these kinases in normal and tumour cells. In addition, the aim was to recreate a normal expression pattern in tumour cells and a transformed expression pattern in normal cells in order to investigate the effects on Apoptin cytotoxicity and to determine a role for PKCβ1 and PKG-I in Apoptin phosphorylation and function.

## *5.2 Results*

### 5.2.1 Inducible expression of PKG-I and its effect on Apoptin phosphorylation

Cell lines SW620 and HT29 and their PKG-I inducible pairs, were treated with Apoptin with or without the presence of their inducible drugs, mifepristone and doxycycline, respectively. Phosphorylation of Apoptin was detected using an antibody specific to T108 phosphorylation and compared to total Apoptin levels using an antibody able to detect Apoptin independently of its phosphorylation status. Using Western blot analysis, levels of phosphorylated Apoptin were compared with total Apoptin levels between induced and non-induced cell lines and the parental cells given the same treatment.

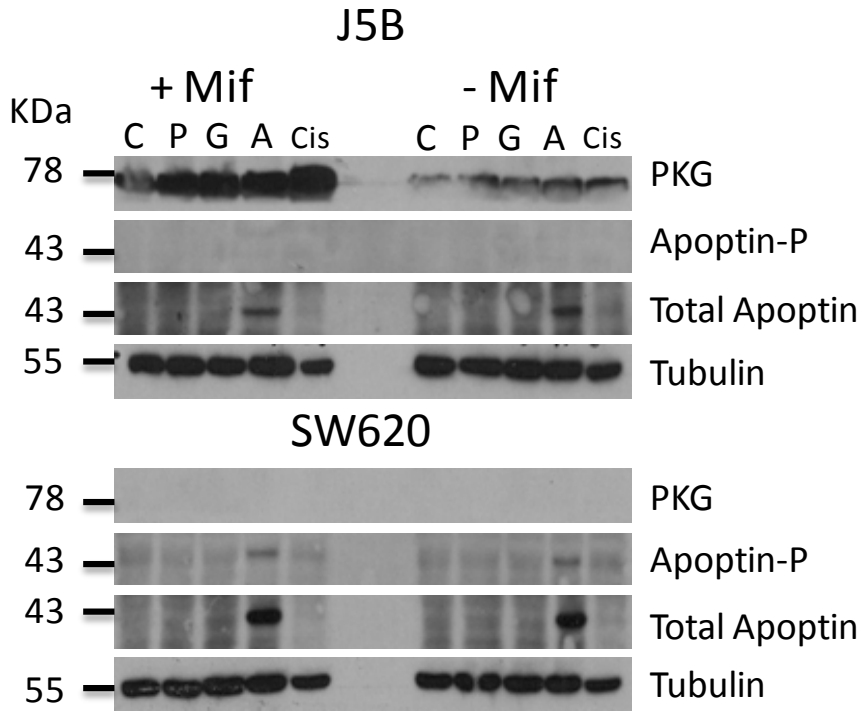
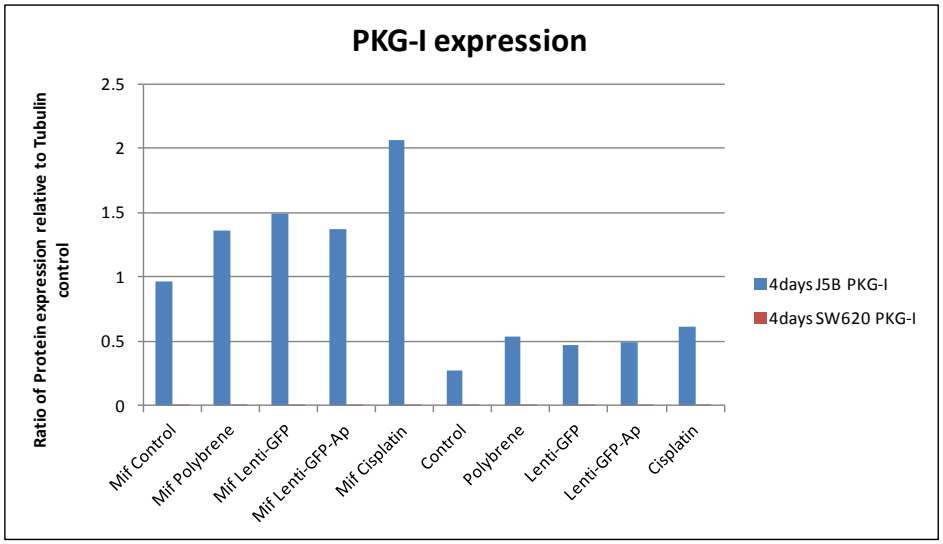



**Figure 5.1 Western blot analysis of PKG-I expression compared to phosphorylated Apoptin in SW620 and J5B.** Cell lines treated with mifepristone and infected with MOI 4 of Lentiviral GFP-Apoptin. Cells were grown for 4 days. C=Control, P=Polybrene treated, G=Lenti-GFP, A=Lenti-GFP-Apoptin, Cis=Cisplatin.

Figure 5.1 shows changes in the expression of PKG-I in the inducible cell line J5B in comparison to the J5B uninduced and parental cell line SW620. Each cell line was treated with mifepristone as control and infected with Lentiviral GFP-Apoptin. In Figure 5.1 and 5.2A, expression of PKG-I was induced by mifepristone in J5B; this effect is not seen in SW620 which showed no detectable levels of PKG-I. However, un-induced J5B also expressed a basal level of PKG-I which could be due to the leaking of the inducible plasmid. In SW620 cells, Apoptin is phosphorylated, with mifepristone treated cells showing an increase in total Apoptin expression and phosphorylated Apoptin expression when the data was quantified (Figure 5.1 and 5.2C). In contrast, J5B cells show no detectable expression of phosphorylated Apoptin when induced by mifepristone and when the cells are untreated (Figure 5.1 and 5.2B); however, the total Apoptin detected was lower than in the SW620 cells. This data indicates that the presence of PKG-I in this cell line affects the expression of Apoptin, decreasing the total level and thereby reducing phosphorylation to undetectable levels in J5B (Figure 5.2B) when compared to the parental SW620 (Figure 5.2C).

A

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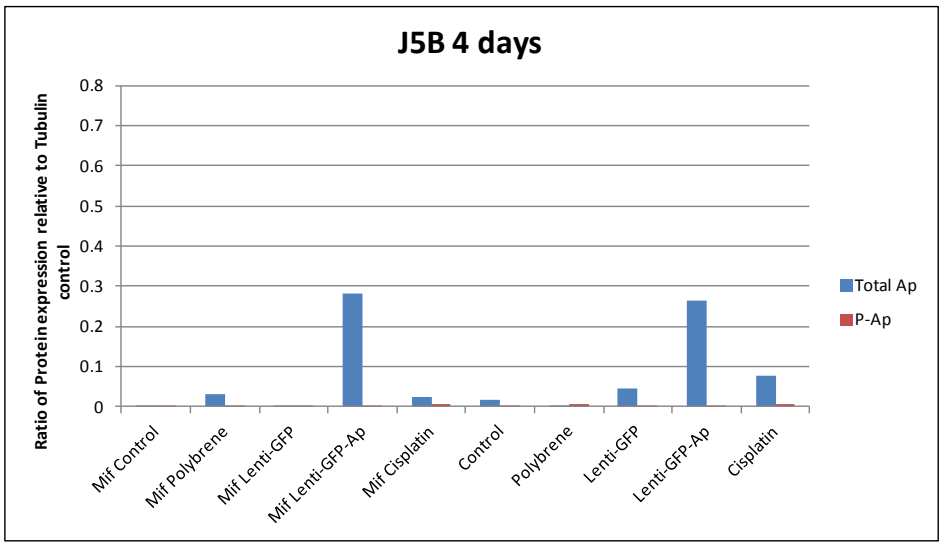



C

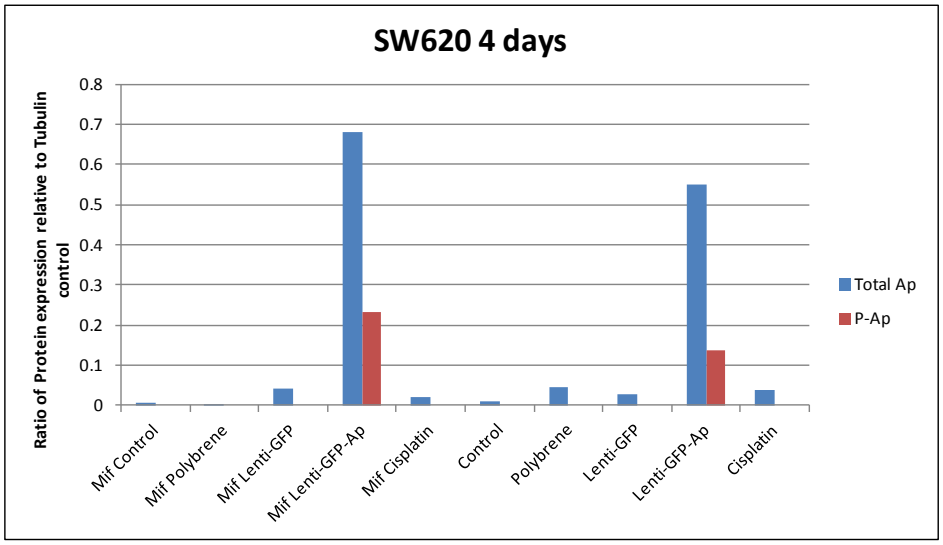



**Figure 5.2 PKG-I expression and Apoptin phosphorylation data quantified for SW620 and J5B**. Cells using imageJ software. Quantification from Western blot data in Figure 5.1.

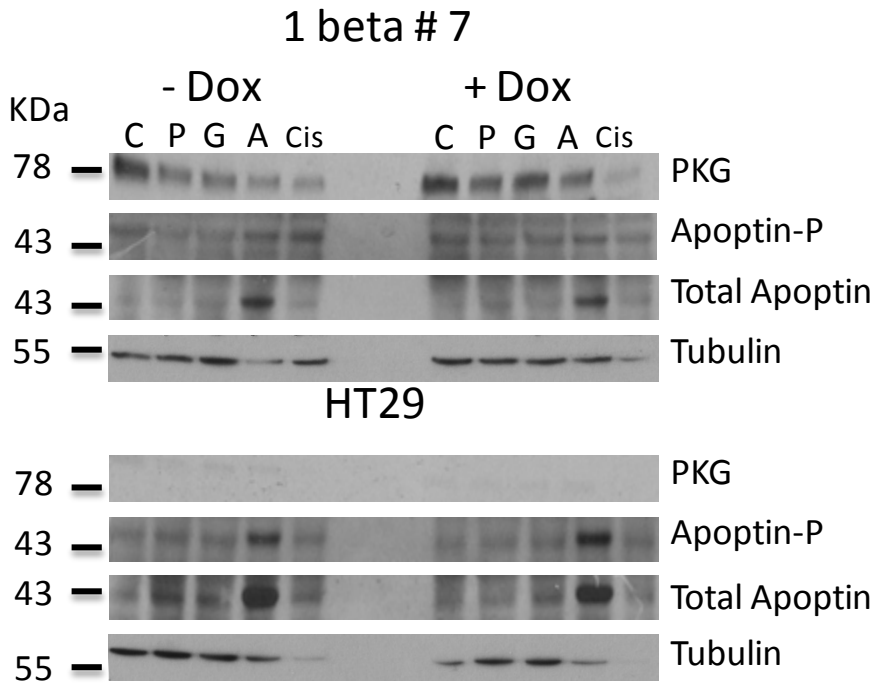



**Figure 5.3 Western blot analysis of PKG-I expression compared to phosphorylated Apoptin in HT29 and 1 beta#7.** Cell lines treated with doxycycline and infected with MOI 4 Lentiviral GFP-Apoptin. Cells were grown for 4 days. C=Control, P=Polybrene treated, G=Lenti-GFP, A=Lenti-GFP-Apoptin, Cis= Cisplatin.

HT29 cells and their doxycycline inducible clones, 1beta#7, were used as a second cell line pair to confirm the previous result with SW620 and J5B, and to investigate if the result was specific only to SW620. As with the SW620 experiment, HT29 cells and their inducible clones were treated with doxycycline before being infected with Lentiviral GFP-Apoptin. Western blot analysis in Figure 5.3 shows a similar pattern to that seen in Figure 5.1, PKG-I expression correlates with reduced phosphorylation of Apoptin. Figure 5.3 indicates that the basal level, likely due to leaking of the inducible system, of PKG-I in the 1beta#7 clone is higher than HT29 as detected by Western blot. Induction of PKG-I expression by doxycycline treatment increased expression of PKG-I in the control and Lentiviral GFP-Apoptin treated 1beta#7 cells, however an increase in expression was not detected in all doxycycline treated variables (Figure 5.4A). This is a marked difference to the J5B system where induced expression was a lot higher than basal levels of PKG-I (Figure 5.2A). The presence of PKG-I expression, however, did correlate with a reduction of Apoptin phosphorylation detected by Western blot (Figure 5.3 and 5.4B and C) and compared to the HT29 parental cells that do not express PKG-I. However, as with the J5B

system, total Apoptin expression was also reduced in the PKG-I expressing cell line, suggesting that PKG-I interferes with Apoptin expression, rather than phosphorylation.

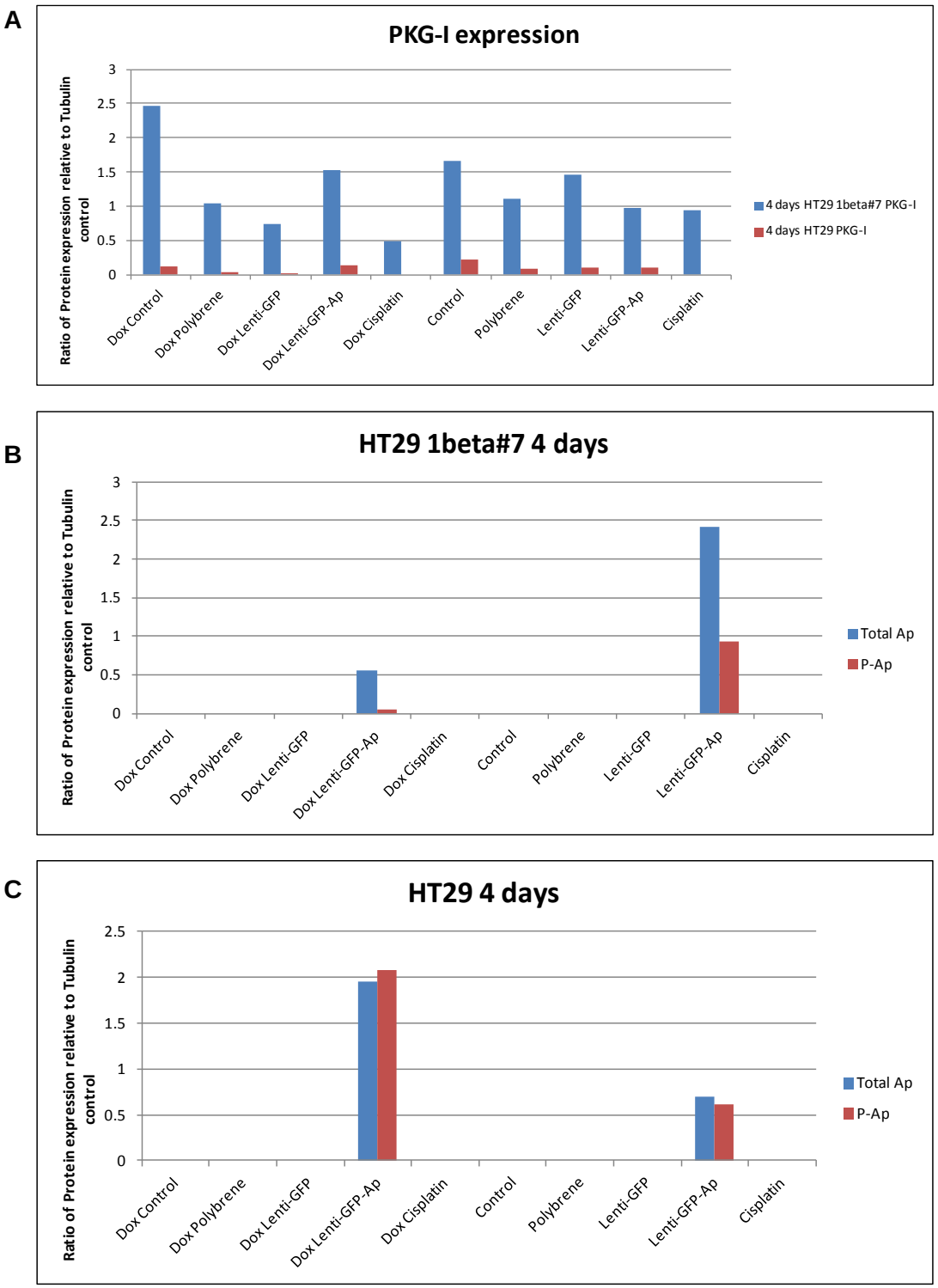



**Figure 5.4 PKG-I expression and Apoptin phosphorylation data quantified for HT29 and 1beta#7.** Analysed using imageJ software. Quantification from Western blot data in Figure 5.3.

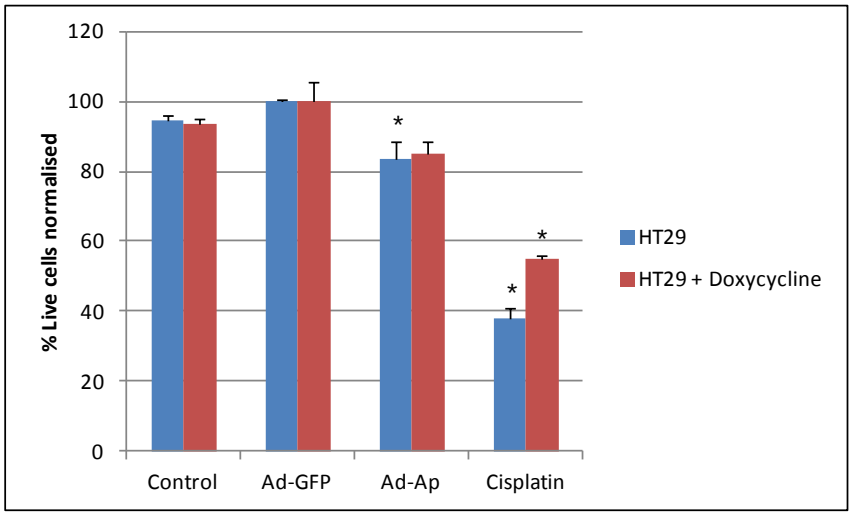
### 5.2.2 Inducible expression of PKG-I and its effect on Apoptin induced cytotoxicity

In the previous results section in the HT29 inducible clone 1beta#7, Apoptin seemed less phosphorylated than the parental HT29 cells. To determine the potential role of this phosphorylation in cytotoxicity a similar experiment was performed and analysed using FACS to investigate cell death. Cells were treated with doxycycline and then infected with Adenoviral vectors expressing Apoptin or control GFP at MOI 40. The cells were treated for 72 hours and collected for FACS analysis.

Data in Figure 5.5A indicate that HT29 cells are sensitive to Apoptin induced cell death with a reduction in live cells of approximately 15%; doxycycline treatment had no impact on Apoptin sensitivity in this cell line. Interestingly, cisplatin treatment was also affected by doxycycline treatment in this cell line, reducing the toxicity of the drug by approximately 15%. Treating the 1beta#7 inducible cells with doxycycline had a similar effect on cisplatin toxicity, with the treated cells showing a reduced sensitivity to the drug (Figure 5.5B). Doxycycline treatment reduced the number of live cells in 1beta#7 and also in the control and Ad-GFP, suggesting a mild toxicity associated with induction of PKG-I in this clone (Figure 5.5B). However, doxycycline treatment of 1beta#7 cells as shown in Figure 5.5B suggests that Ad-Apoptin sensitivity was completely inhibited upon induction of PKG-I expression when compared to the non-treated cells (Figure 5.5B).

Comparing both cell lines with doxycycline treatment, as shown in Figure 5.5C, suggests that only Ad-Apoptin expression in 1beta#7 cells treated with doxycycline exhibits increased resistance to the protein.

A

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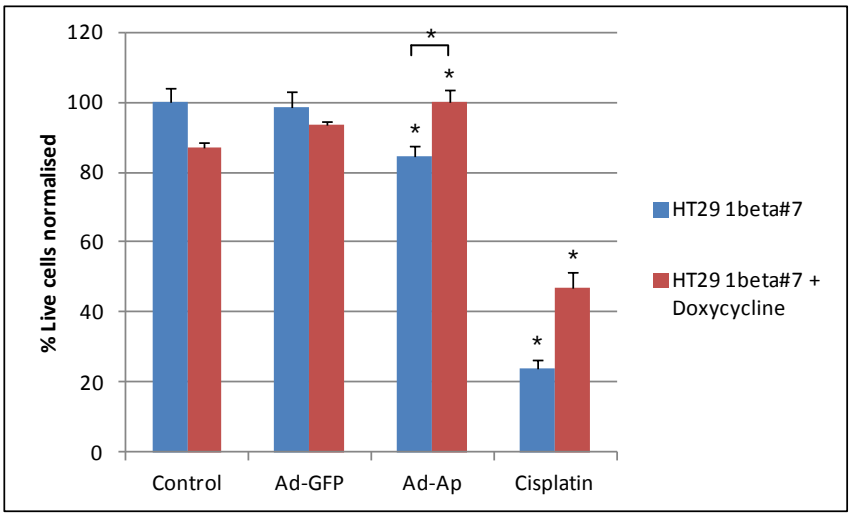


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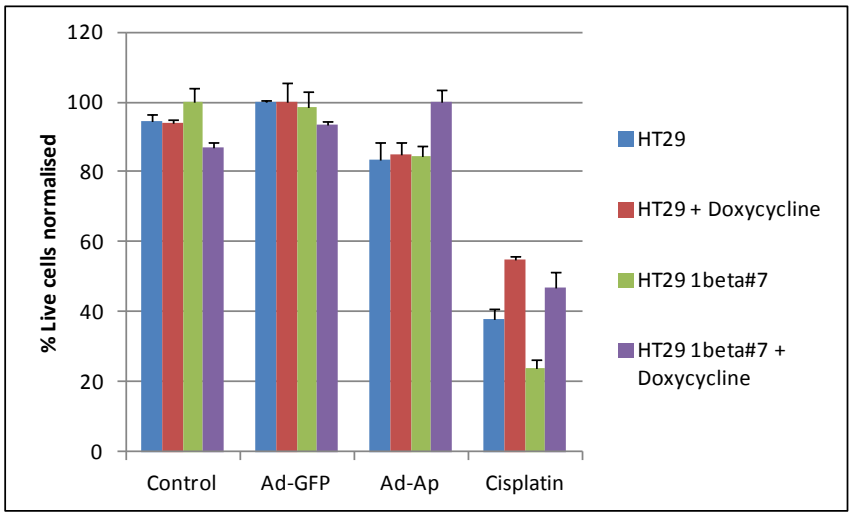



**Figure 5.5 FACS analysis for HT29 and 1beta#7 cells treated with doxycycline inducing agent and infected with Adenoviral vectors for Apoptin and GFP.** Cells were grown for 72 hours. Error bars indicate standard deviation. n=3. Cell death shown here is from the total population of cells treated; this data does not take into account efficiency of adenoviral infection. For example, cells were not gated for Apoptin or GFP positive.

### 5.2.3 Effects of PKCβ1 and PKG-I modulation on Apoptin phosphorylation and sensitivity in colon cancer

Previous results with inducible clones showed that modulation of PKG-I expression affects Apoptin sensitivity and expression. To further investigate the potential role of PKG-I, and also PKCβ1, in Apoptin function HCT116 and NCM460 colon cell lines were used.

As was described in Chapter 4, HCT116 and NCM460 are tumour and normal colon cell lines respectively. Each cell line expressed PKCβ1 and PKG-I kinase with a pattern that correlates with Apoptin sensitivity; HCT116 are sensitive to Apoptin and have high levels of PKCβ1 and low levels of PKG-I (Figure 4.3 and 4.8). In NCM460 the inverse is seen, resistance to Apoptin with low levels of PKCβ1 and high relative levels of PKG-I. To further investigate the importance of these associations, overexpression and siRNA knockdown studies were used.

Figure 5.6 shows the effect of knockdown and overexpression studies in NCM460 cells. The data suggest that PKCβ1 was overexpressed and resulted in an increase in the level of Apoptin phosphorylation when compared to the control cells (Figure 5.6B). Although there appears to be overexpression of PKCβ1, the bands are not clearly defined, so the result is not entirely conclusive. Interestingly, in the PKCβ1 over-expressing cells, PKG-I expression was reduced compared to both control as well as the PKG-I siRNA treated cells. Treatment of cells with siRNA targeting PKG-I, was shown to reduce PKG-I expression when compared to the control siRNA, although PKG-I knockdown was inefficient in the Ad-GFP infected cells. Ad-Apoptin phosphorylation was also marginally increased in the siPKG-I treated cells. Interestingly, the siControl expresses a higher level of phosphorylated Apoptin than both the control and PKCβ1 overexpressing samples, suggesting that the levels of phosphorylated Apoptin can vary between experiments. Additionally, it should be noted that although phosphorylation levels of Apoptin were well detected, the expression of total Apoptin was very low with a high level of background (Figure 5.6A).

Using HCT116 cells, PKCβ1 was knocked down and PKG-I was overexpressed to determine changes in the phosphorylation of Apoptin (Figure 5.7). As shown in Figure 5.7A, overexpression of PKG-I resulted in a decreased level of PKCβ1 and, against expectations; these cells expressed a higher level of phosphorylated Apoptin

compared to control (Figure 5.7B). However, the control sample expressed total Apoptin at a much lower level than the PKG-I overexpressing sample. PKCβ1 knockdown by siRNA in HCT116 cells decreased the level of expression of PKCβ1 by approximately half compared to the control siRNA, however this resulted in no effect on the phosphorylation of Apoptin compared to siControl (Figure 5.7B).

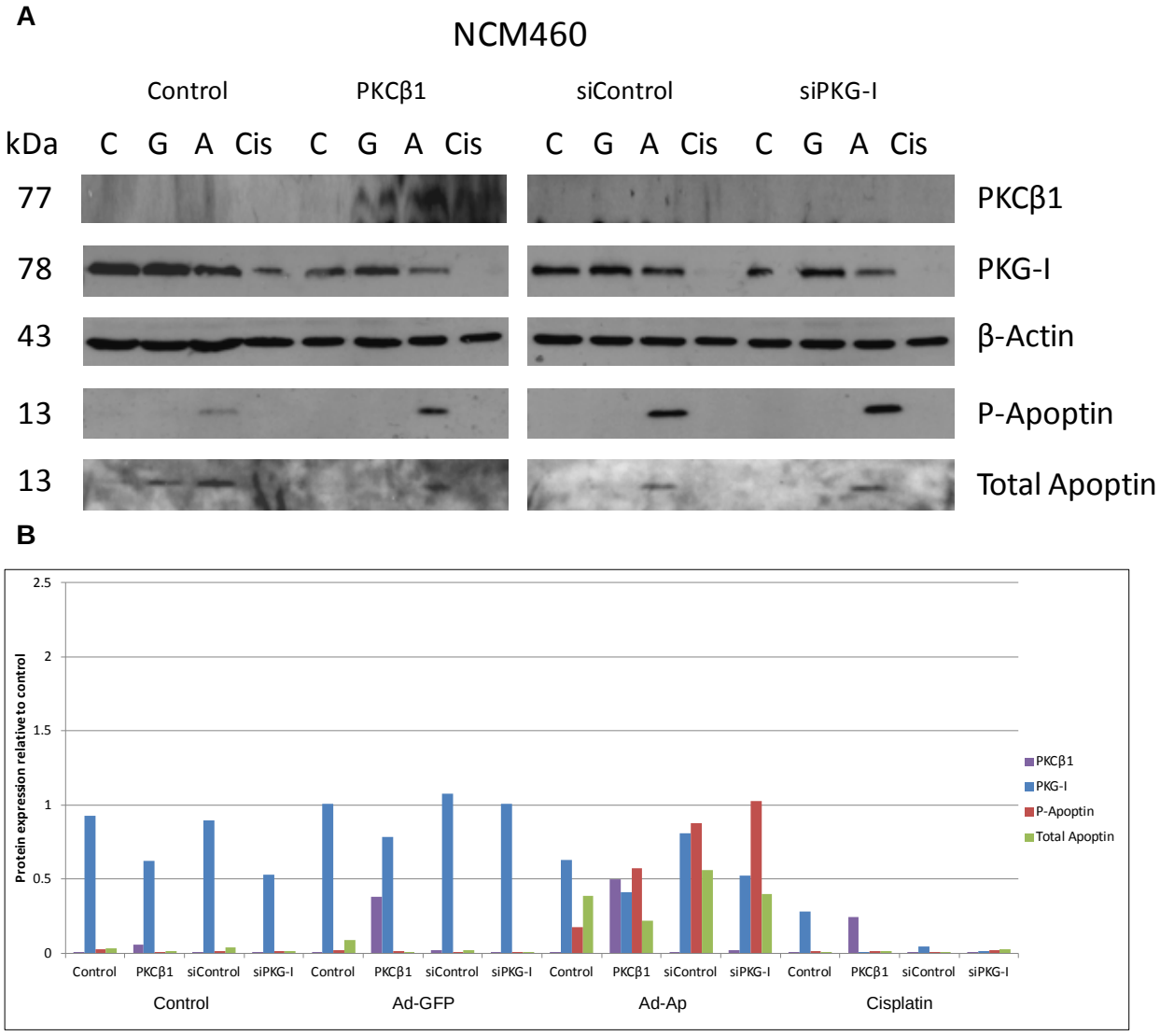



**Figure 5.6 Western blot analysis of PKCβ1, PKG-I and Apoptin expression in NCM460.** Displaying overexpression of PKCβ1 using plasmid transfection and knockdown of PKG-I using siRNA and the effect on Apoptin phosphorylation. Western blot data (A) and quantification using imageJ software (B).

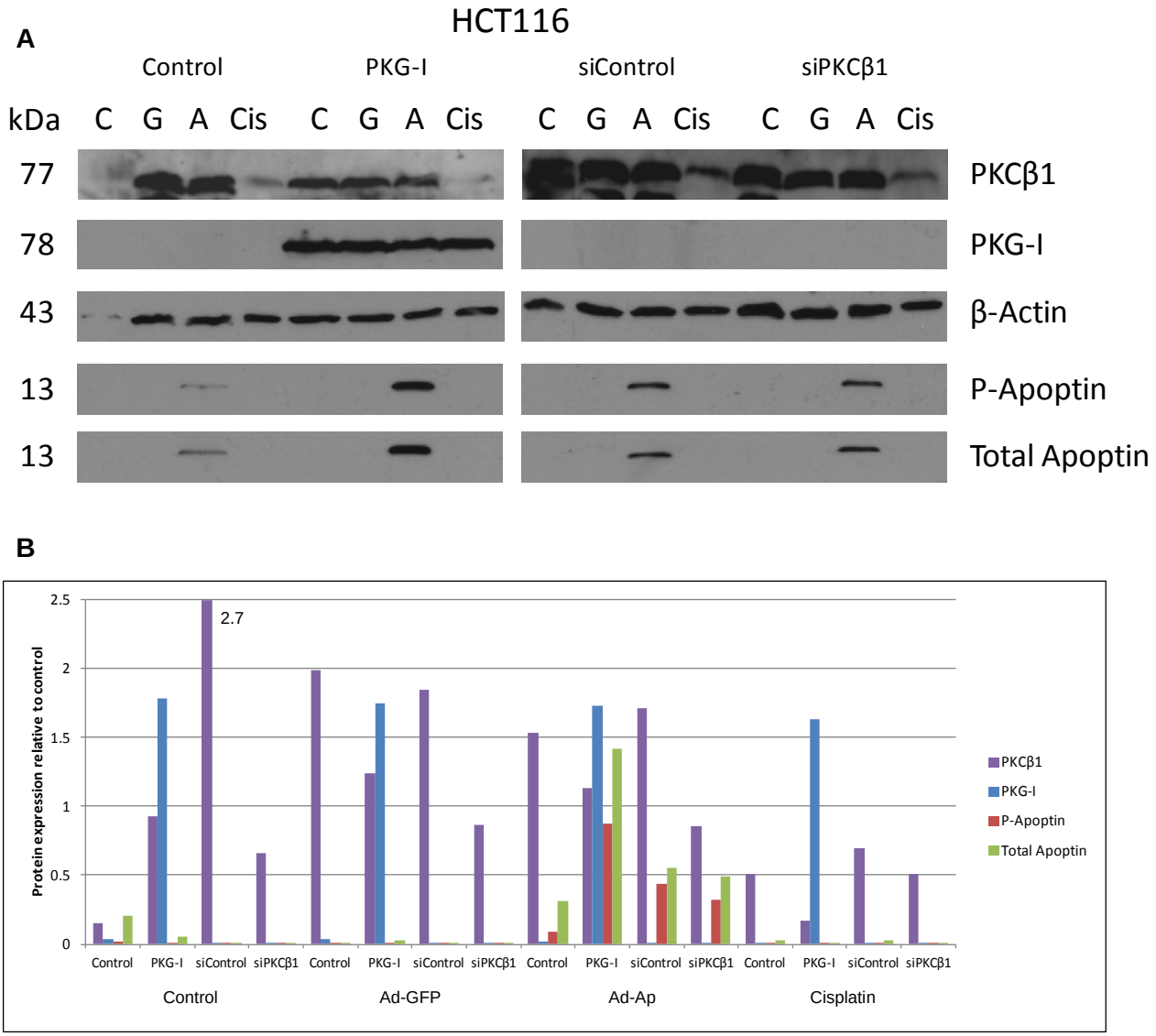


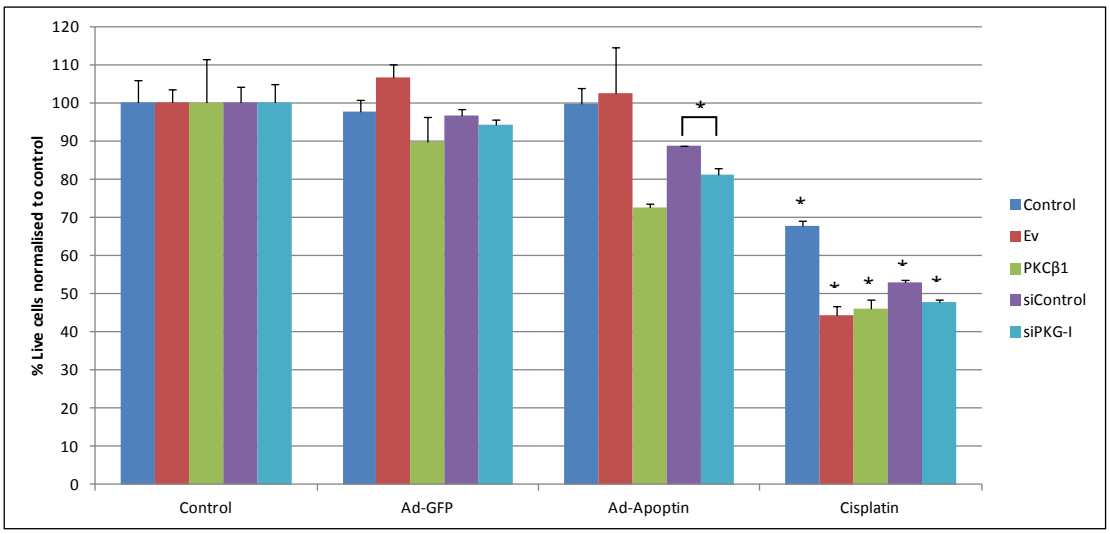

**Figure 5.7 Western blot analysis of PKCβ1, PKG-I and Apoptin expression in HCT116.** Displaying overexpression of PKG-I using plasmid transfection and knockdown of PKCβ1 using siRNA and the effect on Apoptin phosphorylation. Western blot data (A) and quantification using imageJ software (B).

Using FACS analysis, cells were treated with siRNA or transfected with plasmids for expression of PKCβ1 or PKG-I exogenously, and then infected with Adenoviral vectors expressing either Apoptin or GFP as a control. The results of these experiments as shown in Figure 5.8A, suggested that for NCM460 cells overexpression of PKCβ1 made these cells more sensitive to Apoptin induced cell death with a reduction in viable cells of approximately 30%. PKG-I siRNA treatment resulted in a 20% loss of cell viability with Apoptin treatment as compared to a 10% loss of viability in cells treated with siRNA control. Cisplatin treatment showed a similar loss of live cells across each variable, with Control cells displaying the smallest loss in viable cells.

For HCT116 cells, the FACS data, shown in Figure 5.8B, revealed that upon infection with Ad-Apoptin, empty vector transfected cells lost viability of 40%. When cells overexpressed PKG-I, the loss of viability was reduced to 20%, although this was not a reduction in cell death when compared to untreated control cells. Knockdown of PKCβ1 was shown to reduce Apoptin induced cell death when compared to all controls except the siRNA control, where the difference was minimal. Cisplatin treatment, again, showed a similar loss of cell viability as described for NCM460 cells (Figure 5.8B).

Collectively these functional studies suggest that interference with PKG-I and PKCβ1 expression appears to correlate with Apoptin sensitivity in the normal NCM460 colon cells, but this correlation does not seem to extend to the transformed HCT116 colon cells. Normal cells expressing PKG-I at high levels and expressing low levels of PKCβ1 are less sensitive to Apoptin induced cell death compared to tumour cells expressing high levels of PKCβ1 and lower relative levels of PKG-I; however this observation, described in Chapter 4 is based on endogenously expressed levels of each kinase. Exogenous overexpression of PKG-I in HCT116 cells may not have the same effect as the endogenous expression associated with the normal NCM460 colon cells.

**A**




**B**

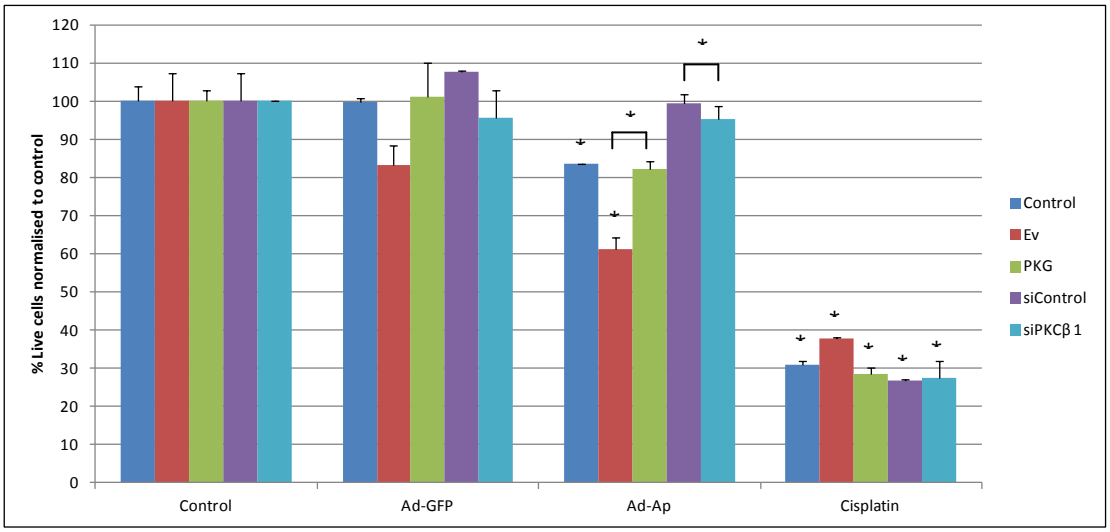



**Figure 5.8 FACS analysis of colon cell lines NCM460 and HCT116 and their sensitivity to Apoptin.** Delivered by Adenovirus at MOI 40 after knockdown or overexpression of PKCβ1 and PKG-I. NCM460 (A) and HCT116 (B). Cells were treated for 72 hours. Error bars indicate standard deviation. Data from at least 2 separate experiments. Cell death shown here is from the total population of cells treated; this data does not take into account efficiency of adenoviral infection. For example, cells were not gated for Apoptin or GFP positive expression.

### 5.2.4 Effects of PKCβ1 and PKG-I modulation on Apoptin phosphorylation and sensitivity in a transformed fibroblast model

1BR3 and their transformed isogenic match 1BR3LT were previously shown to differentially express both PKCβ1 and PKG-I (Figure 4.5) and this correlated with differences in Apoptin sensitivity (Figure 4.9). Although these differences were not as marked as those seen with the colon model, it was important to examine whether the fibroblast cell model displayed similar characteristics in relation to the expression of these kinases. To investigate these differences and their potential effects on Apoptin function, siRNA and overexpression studies were performed and the effects were examined using Western blot and FACS analysis.

Overexpression of PKCβ1 in 1BR3 cells was shown to be successful and produced a marked increase in the expression of this kinase. Overexpression of PKCβ1 resulted in a substantial decrease in PKG-I expression compared to control (Figure 5.9) as previously observed with the colon cell lines (Figure 5.6). Knockdown of PKG-I, however, did not appear to have any effect on PKCβ1 expression as detected by Western blot analysis (Figure 5.9A and B). 1BR3 cells that overexpressed PKCβ1 had a reduced level of Apoptin phosphorylation and an overall reduction in the level of total Apoptin expression in contrast with the proposed hypothesis (Figure 5.9B). Further, knockdown of PKG-I had no effect on the level of Apoptin phosphorylation when compared to the siRNA control treated cells, but an increased expression of total Apoptin was observed, also against expectation (Figure 5.9B).

1BR3LT cells were studied in a similar way, with overexpression of PKG-I and siRNA targeted knockdown of PKCβ1 (Figure 5.10). The results show that overexpression of PKG-I induced a marked decrease in the expression of PKCβ1 to a much higher extent than that achieved by PKCβ1 siRNA (Figure 5.10A and B). siRNA knockdown in 1BR3LT did not successfully reduce the expression of PKCβ1 when compared to the siRNA control (Figure 5.10B). Quantification analysis showed that overexpression of PKG-I resulted in reduced Apoptin phosphorylation compared to the control, however, total Apoptin levels were undetectable (Figure 5.10B). In the siPKCβ1 treated cells Apoptin phosphorylation was essentially unchanged compared to the siRNA control (Figure 5.10B).

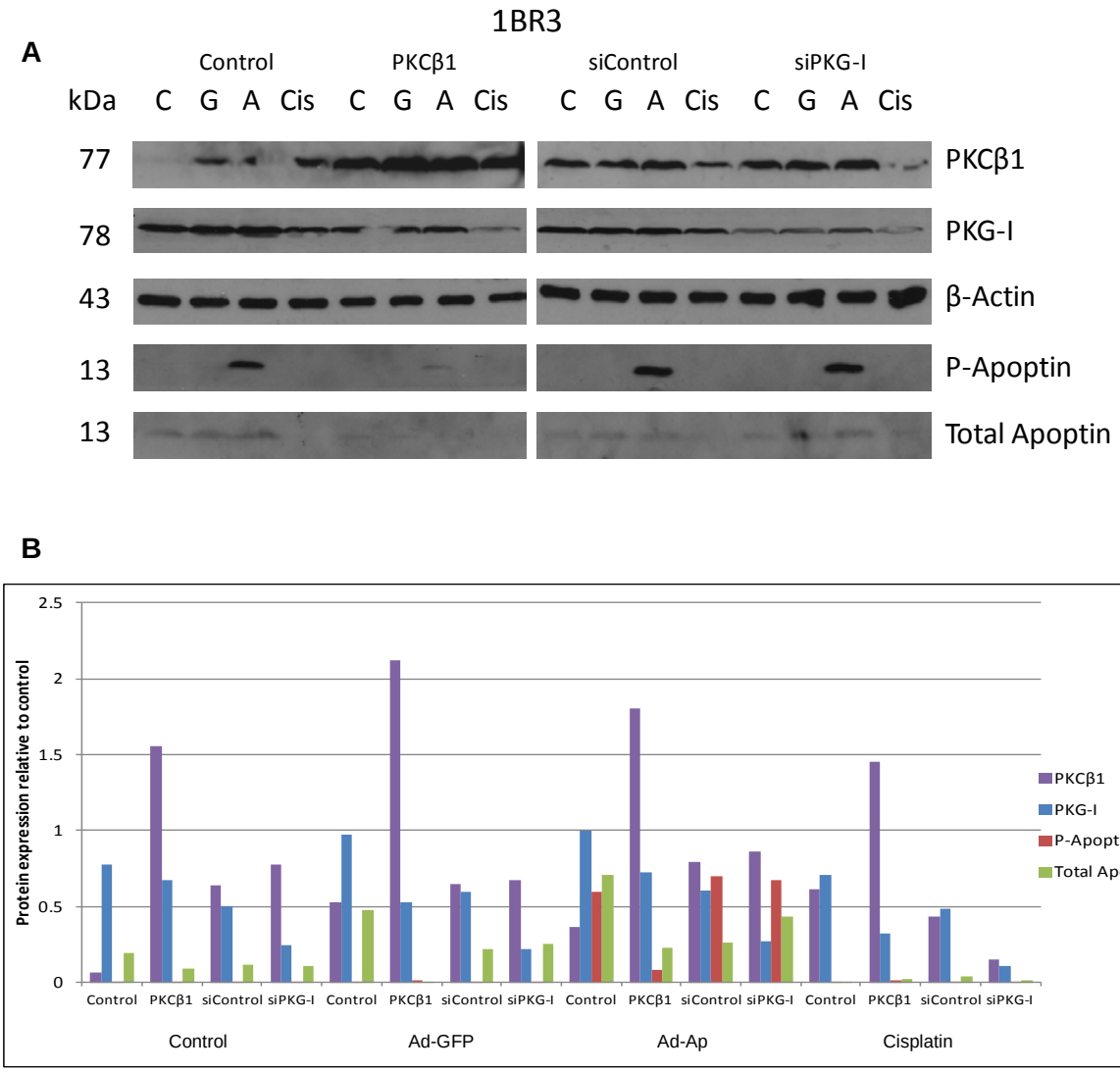



**Figure 5.9 Western blot analysis of PKCβ1, PKG-I and Apoptin expression in 1BR3.** Analysis of 1BR3 after overexpression of PKCβ1 using plasmid transfection and knockdown of PKG-I using siRNA and the effect on Apoptin phosphorylation. Western blot data (A) and quantification using imageJ software (B).

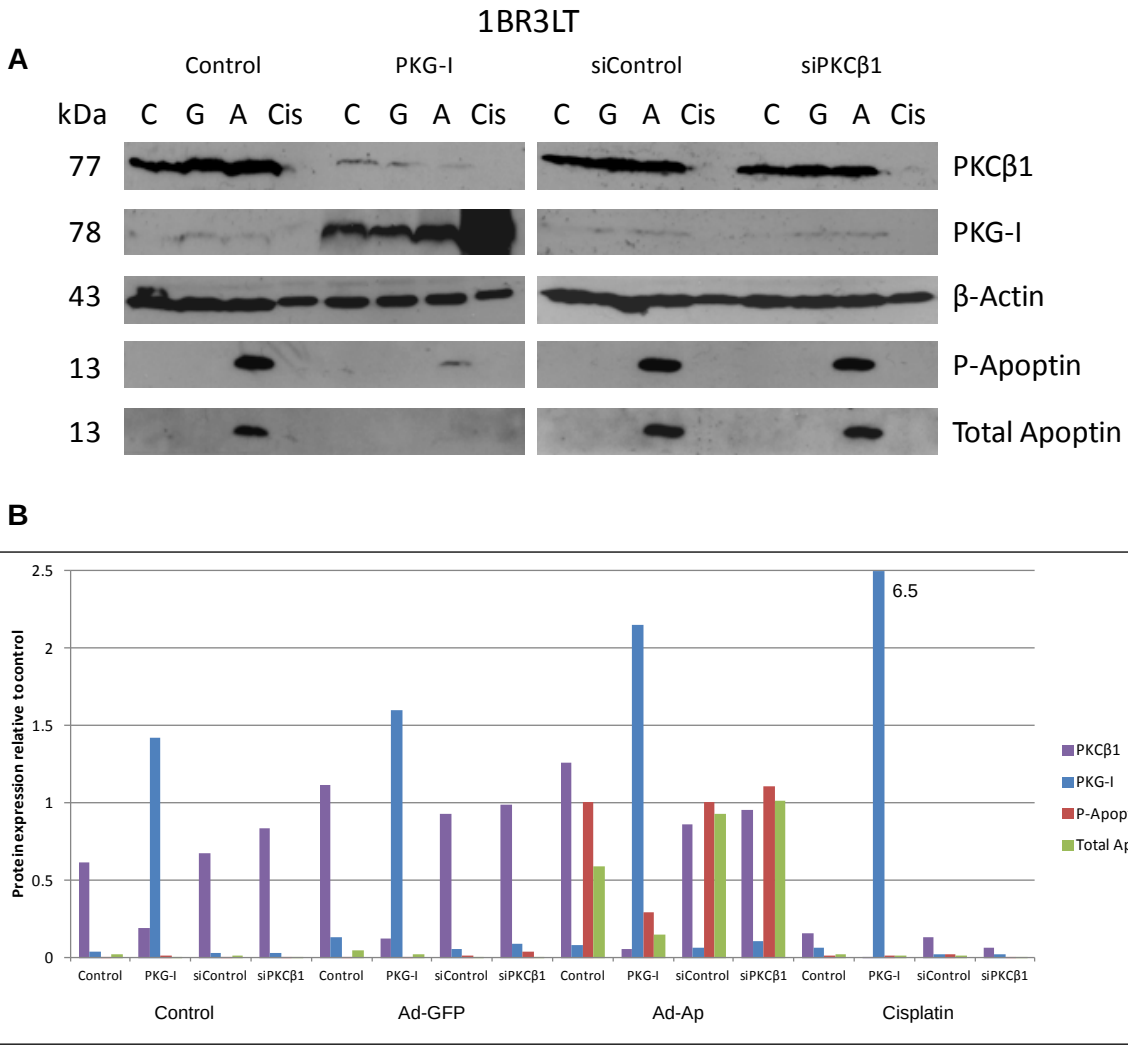



**Figure 5.10 Western blot analysis of PKCβ1, PKG-I and Apoptin expression in 1BR3LT.** Analysis of 1BR3LT after overexpression of PKG-I using plasmid transfection and knockdown of PKCβ1 using siRNA and the effect on Apoptin phosphorylation. Western blot data (A) and quantification using imageJ software (B).

To further investigate the role of PKCβ1 and PKG-I in Apoptin function, sensitivity to Apoptin induced cytotoxicity was investigated in relation to kinase expression by FACS analysis.

The results in Figure 5.11A show that PKCβ1 overexpression had no effect on Apoptin induced cell death when compared to the controls; the cells responded in a similar way to the empty vector control transfected cells, with no increase in cell death upon Apoptin treatment. In the cisplatin treated cells, the PKCβ1 expressing cells again showed no variation in cell death compared to empty vector control, however, a reduction of 30% in cell viability was observed as compared to non-transfected controls (Figure 5.11A). Knockdown by siRNA of PKG-I resulted in a significant increase in the percentage of cell death in Apoptin treated cells of approximately 10% compared to the siRNA PKG-I untreated cells (Figure 5.11A). Cisplatin treatment induced an increased cell death compared to siRNA control and untreated control cells; however, this cell death was comparable to empty vector and PKCβ1 transfected cell lines (Figure 5.11A).

1BR3LT, the transformed isogenic match of 1BR3 cells, were used to test the sensitivity of Apoptin in relation to the expression of these kinases and compare to their matched normal cells described above. Figure 5.11B shows that upon the induction of PKG-I overexpression, 1BR3LT cells became more sensitive to Apoptin induced cytotoxicity, with a small reduction in cell viability of 5% compared to the control. Cisplatin treatment showed no change in cell death compared to both the control and empty vector control. Knockdown of PKCβ1 results showed that siRNA knockdown was not successful in 1BR3LT (Figure 5.10). As a result of this, no change in Apoptin sensitivity was observed (Figure 5.11B).

In the normal versus transformed fibroblast model, PKG-I expression appears to have some effect on Apoptin sensitivity, but there is no observable effect of PKCβ1 expression on Apoptin function.

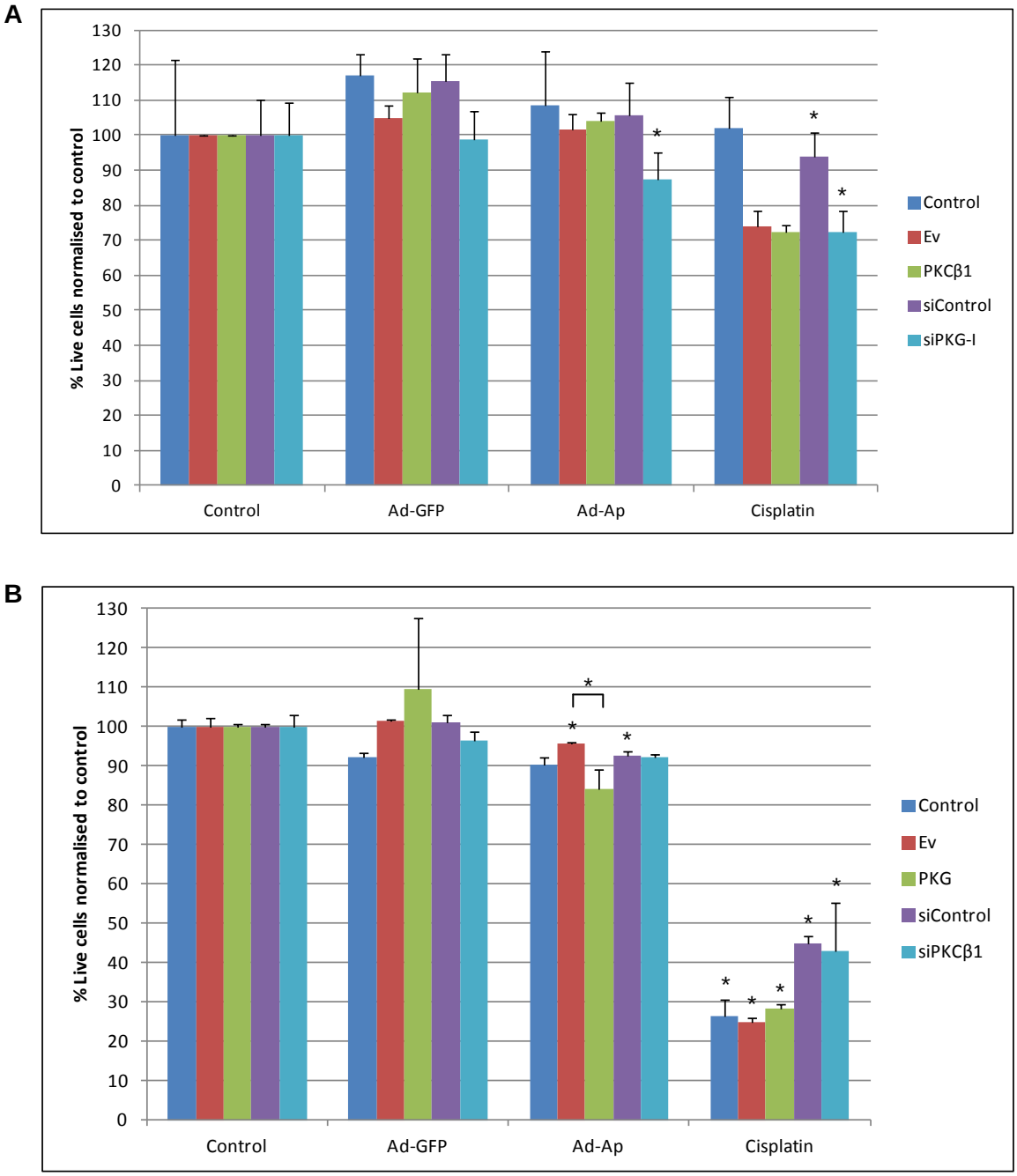



**Figure 5.11 FACS analysis of fibroblast cell lines 1BR3 and 1BR3LT and their sensitivity to Apoptin.** Delivered by Adenovirus at MOI 40 after knockdown or overexpression of PKCβ1 and PKG-I. 1BR3 (A) and 1BR3LT (B). Cells were treated for 72 hours. Error bars indicate standard deviation. Data from at least 2 separate experiments. Cell death shown here is from the total population of cells treated; this data does not take into account efficiency of adenoviral infection. For example, cells were not gated for Apoptin or GFP positive expression.

### 5.2.5 Effects of PKCβ1 and PKG-I modulation on Apoptin cellular localisation

In the previous sections PKCβ1 and PKG-I expression was found to correlate with the phosphorylation and cytotoxic effects of Apoptin. The tumour specific nature of Apoptin has been linked to many specific characteristics and behaviours in normal and transformed cells, one of which being subcellular localisation (Guelen et al., 2004). To determine whether modulation of the kinases described in this study affected Apoptin localisation in addition to its phosphorylation and cytotoxicity, immunofluorescence studies were performed.

Using HCT116, NCM460 and 1BR3 cell lines, a combination of siRNA and plasmid overexpression techniques were used as described in previous sections, followed by infection with Adenovirus to express Apoptin or GFP control and then the cellular localisation was analysed.

HCT116 cells were transfected with siRNA targeted to PKCβ1 and changes in Apoptin localisation were observed as described in Figure 5.12A. The results showed that when PKCβ1 expression is reduced, HCT116 cells displayed healthier looking nuclei in Apoptin expressing cells compared to siRNA control cells (Figure 5.12A). There is also a reduction in the number of cells expressing Apoptin after treatment with siRNA against PKCβ1 in Figure 5.12A.

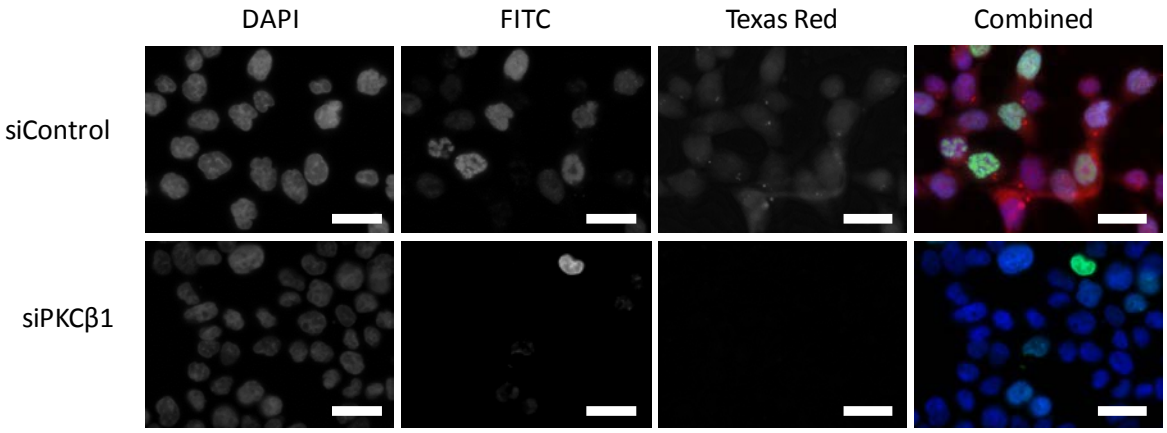
NCM460 cells were also transfected with siRNA, but this time targeted to PKG-I. In those cells treated with PKG-I siRNA, Apoptin appeared to localise at a higher level in the nucleus compared to the siRNA control, with aggregates of Apoptin within the nucleus of these cells (Figure 5.12B). This correlated with increased Apoptin sensitivity results in NCM460 upon knockdown of PKG-I as was shown in Figure 5.8A. NCM460 cells were also transfected to overexpress PKCβ1 to determine the effects on localisation of Apoptin, as there was a significant change in Apoptin sensitivity as shown in Figure 5.8A. The results in Figure 5.12B showed that overexpression of PKCβ1 in NCM460 cells and subsequent Apoptin infection induced a noticeable increase in the level of Apoptin nuclear aggregates as compared to the empty vector control. This aggregation produced an expression pattern indicative of PML body

localisation which, interestingly, is associated with the binding and localisation of Apoptin in tumour cells (Poon et al., 2005).

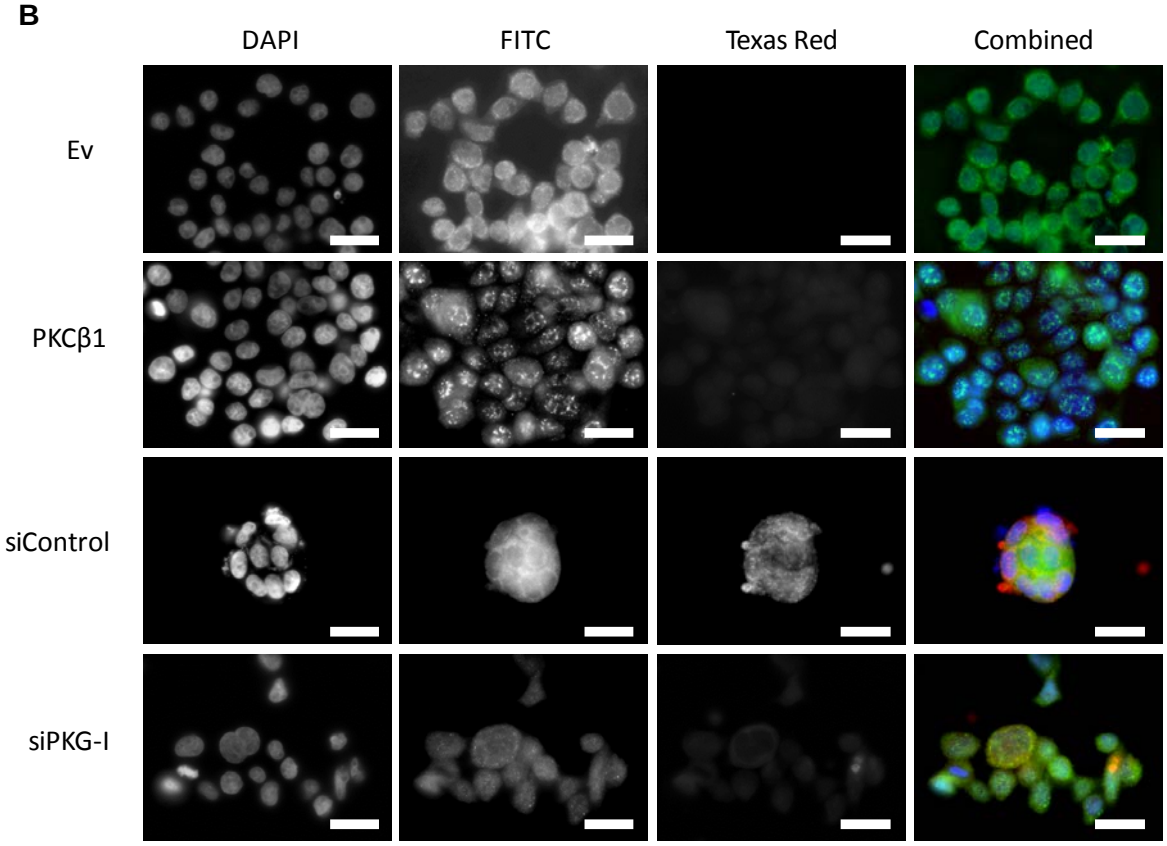
1BR3 cells were transfected in a similar way to NCM460 with knockdown of PKG-I by siRNA and overexpression of PKCβ1 using plasmid transfection. Results in Figure 5.12C show that when PKCβ1 is overexpressed in 1BR3 cells, Apoptin localises more in the nucleus compared with cells transfected with the empty vector control, however, there is still cytoplasmic localisation seen in these cells. This could potentially be responsible for the lack of sensitivity to Apoptin induced cytotoxicity seen in Figure 5.11A. In contrast, knockdown of PKG-I in 1BR3 cells was shown to increase Apoptin sensitivity (Figure 5.11A). Figure 5.12A shows that siRNA knockdown of PKG-I in 1BR3 cells induced a dramatic change in Apoptin localisation compared to siRNA control. Control cells display characteristic cytoplasmic localisation of Apoptin, but once PKG-I expression is abrogated Apoptin localises to the nucleus with little or no Apoptin detected in the cytoplasm.

The above results suggest that PKCβ1 and, more importantly, PKG-I expression could potentially be linked to Apoptin localisation in the cell lines tested.

**A**


DAPI
FITC
Texas Red
Combined
siControl
siPKCβ1

**B**


DAPI
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Ev
PKCβ1
siControl
siPKG-I

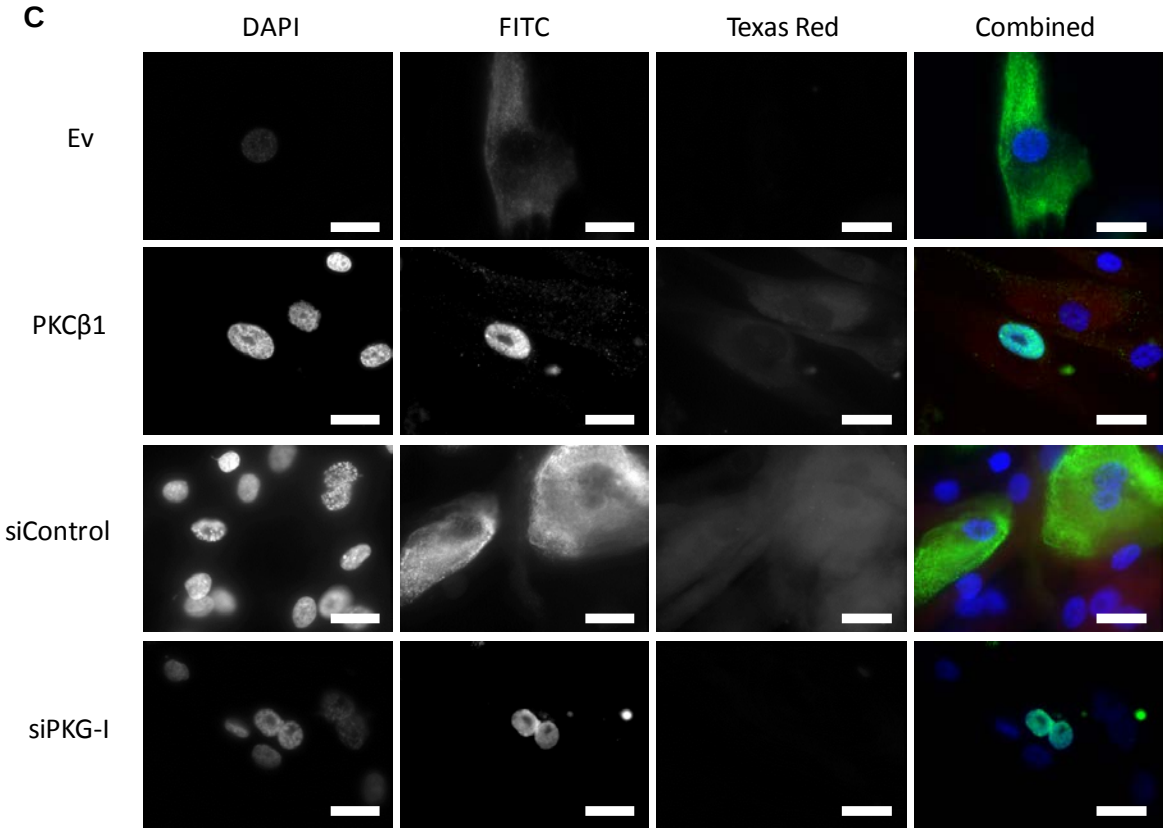


**Figure 5.12 Immunofluorescence images of HCT116, NCM460 and 1BR3 cells showing Apoptin localisation.** Immunofluorescence images of HCT116 (A), NCM460 (B) and 1BR3 (C) cells infected with Adenoviral Apoptin after overexpression or knockdown of PKCβ1 or PKG-I. Apoptin was visualised with FITC conjugated antibodies, and PKCβ1 or PKG-I were visualised with Texas Red conjugated antibodies. Nuclei visualised with DAPI staining. 60x magnification. Scale bar: 25μm.

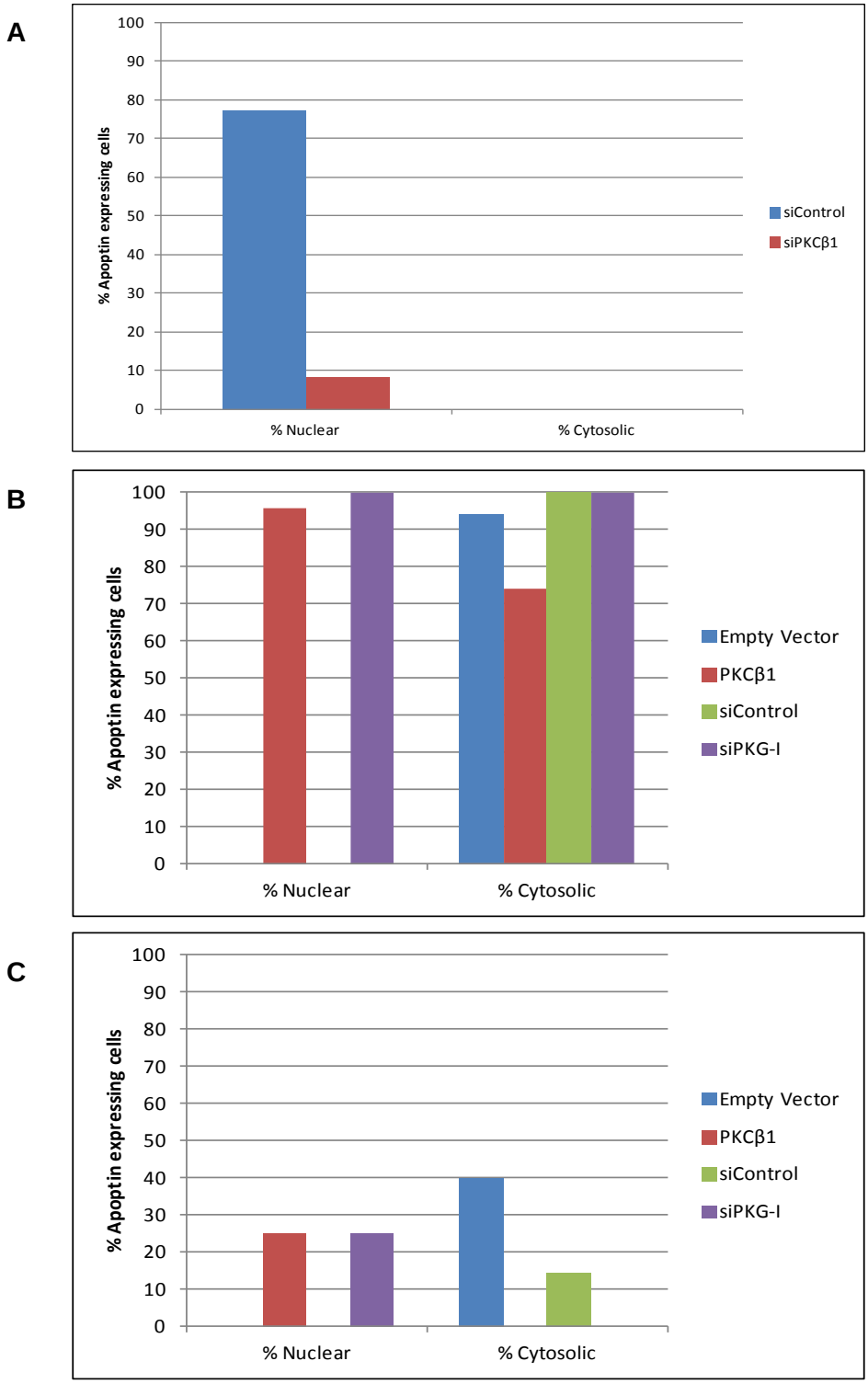


**Figure 5.13 Quantification of cellular localisation of Apoptin in HCT116 (A), NCM460 (B) and 1BR3 (C) cells.** Quantification of nuclear and cytosolic localisation of Adenoviral Apoptin taken by manually counting Apoptin expressing cells from the experiment shown in Figure 5.12. Only one cell count was possible for each cell type.

## *5.3 Discussion and Conclusions*

Based on the observations made in Chapter 4, the aims of this section were to modify expression of PKCβ1 and PKG-I and further investigate the effects of these changes on the function of Apoptin in paired normal and transformed cell lines.

SW620 and their inducible clone, J5B, were tested for inducible PKG-I expression and the effect on Apoptin phosphorylation. Interestingly, PKG-I expressing clones, with or without induction were shown to express Apoptin with no detectable phosphorylation (Figure 5.1 and 5.3), however, total Apoptin expression was also reduced. This result suggests that PKG-I expression may in some way interfere with Apoptin phosphorylation in these cell lines, but could also affect the total expression of Apoptin. Abrogated phosphorylation was also seen in the non-induced clones; however, these cells did express some PKG-I that was at a much higher level than that seen in the parental cell lines. This is likely to be due to the leakiness of the inducible system used (Figure 5.2 and 5.4).

Further investigation of the effects of PKG-I expression in the HT29 paired cell lines revealed that the abrogation of Apoptin phosphorylation in the inducible clones also coincided with an increased resistance to Apoptin induced cell death (Figure 5.5B and C). The use of the PKG-I inducible system in this study revealed that there was a correlation between PKG-I expression and Apoptin expression in this model.

The results in Chapter 4 showed a correlation between Apoptin sensitivity and expression of both PKG-I and PKCβ1 in the colon cell model. Thus supporting the notion that normal cells express higher levels of PKG-I (Karami-Tehrani et al., 2012) and lower levels of PKCβ1 compared to tumour cells. This was confirmed to be the case in NCM460 and HCT116 cells, Figure 4.3 and 4.8, by further investigation during this study showing that the silencing of PKG-I by siRNA transfection can result in significant increased Apoptin sensitivity in NCM460 cells compared to siControl cells (Figure 5.8A). However, overexpression of PKG-I in HCT116 cells was less conclusive (Figure 5.8B), suggesting that PKG-I endogenous expression is more significant than exogenous expression in this model and would be an ideal method of further investigation.

NCM460 cells became more sensitive to Apoptin induced apoptosis upon overexpression of PKCβ1 (Figure 5.8A) accompanied by an increase in Apoptin phosphorylation levels (Figure 5.6). Together this data further supports our previous findings that this kinase might be involved in Apoptin phosphorylation and its toxic effects. As previously reported, PKCβ1 phosphorylates Apoptin and induces cell death in cells expressing high levels of PKCβ1 (Jiang et al., 2010a). In HCT116 cells, the effects of PKCβ1 on Apoptin function were less conclusive. PKCβ1 siRNA silencing studies revealed an increased sensitivity to Apoptin by only 5% (Figure 5.8B), however, the siRNA was not very effective and PKCβ1 expression was still detected (Figure 5.7). Although the knockdown was successful in reducing the level of expressed PKCβ1 protein, this was likely not enough to significantly negate the effect this kinase has on Apoptin function in HCT116 cells.

In the 1BR3 model, PKG-I expression was shown to have an effect on the sensitivity of each cell line to Apoptin induced cytotoxicity. For example, 1BR3 cells transfected with siRNA targeting PKG-I were found to be more sensitive to Apoptin. Interestingly, overexpression of PKG-I in the transformed 1BR3LT cell line increased the sensitivity to Apoptin induced cell death. However, studies have shown that the overexpression of PKG in some transformed cell lines can induce apoptosis (Fallahian et al., 2012; Fallahian et al., 2011), possibly explaining the effect seen in 1BR3LT. SV40LT transformation of cell lines has also been shown to reduce expression of PKG; this could explain the reduction in PKG expression seen in 1BR3LT cells. This loss of PKG expression may be required for transformation through this mechanism and reintroduction of PKG through overexpression could, perhaps induce growth arrest and apoptosis (Fujii et al., 1995b).

Overexpression of PKCβ1 in 1BR3 cells was successful, but no effect on Apoptin sensitivity was observed; 1BR3 cells have a basal level of PKCβ1 and an increase in expression may not have an increased effect over this basal activity. In 1BR3LT knockdown experiments for PKCβ1, reduction of expression of PKCβ1 was limited and an overall effect on Apoptin sensitivity was un-noticeable, phosphorylation was also unaffected. From the results obtained here, it can be concluded that PKG-I has some influence on both Apoptin phosphorylation and cytotoxicity in all the cell lines studied. However, PKCβ1 can only be linked to Apoptin phosphorylation and toxicity in the colon model.

Further to these findings it is interesting to note that, in all cell lines tested, overexpressing either PKG-I or PKCβ1 resulted in reduced expression of PKCβ1 or PKG-I, respectively, suggesting a link between the two kinases as has been proposed in previous studies (Hou et al., 2003; Zhu et al., 2009), for example one kinase may regulate the function and/or the level of the other.

In order to fully investigate the effects of PKG-I and PKCβ1 expression on Apoptin and its function, immunofluorescent localisation studies were performed. HCT116 cells were transfected with siRNA targeting PKCβ1 as previously described, and the cellular localisation of Adenovirus delivered Apoptin was analysed. The data showed that although knockdown did not have a major effect on Apoptin sensitivity, compared to the siRNA control there was a reduction in the number of cells expressing Apoptin in the nucleus, however, this coincided with a reduction in total Apoptin expression (Figure 5.12A).

NCM460 cells overexpressing PKCβ1 showed that Apoptin localisation was clearly affected. Every cell analysed presented a changed distribution of Apoptin (Figure 5.12B), indicating that overexpressing PKCβ1 in this cell line causes Apoptin to localise to the nucleus, and subsequently induces cell death. A similar effect is seen upon the knockdown of PKG-I which resulted in Apoptin aggregating in the nucleus of the cell and the appearance of abnormal nuclei. Interestingly, in both cases Apoptin expression increased in the nucleus but there was still evidence of cytosolic expression, suggesting that the nuclear export of Apoptin was still functioning, although not as effectively (Figure 5.13B).

Similarly, the overexpression of PKCβ1 in 1BR3 resulted in Apoptin moving from the cytoplasm into the nucleus of the cells. Cells with successful knockdown of PKG-I exhibited complete nuclear localisation of Apoptin with evidence of nuclear aggregation (Figure 5.13C). This distribution could explain why PKG-I knockdown is important in increasing Apoptin sensitivity in 1BR3 cells.

In conclusion, the data described here suggest a novel role for PKG-I and PKCβ1 in the regulation of Apoptin phosphorylation, localisation and induction of cell death in colon cells. PKG-I abrogates Apoptin expression and interrupts its nuclear shuttling in the cell, which may subsequently lead to resistance to Apoptin induced apoptosis, possibly through the regulation of PKCβ1 (Figure 5.14).

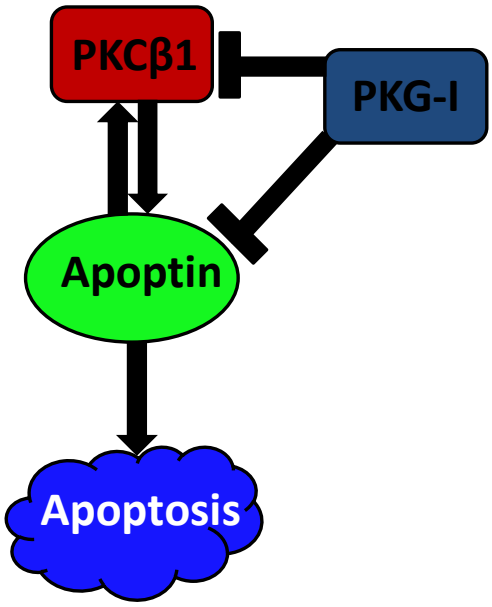


**Figure 5.14 Schematic diagram describing a model of regulation by PKG-I of both Apoptin and PKCβ1.** PKCβ1 phosphorylates Apoptin leading to apoptosis. PKG-I inhibits phosphorylation of Apoptin by PKCβ1 and inhibits Apoptin induced apoptosis.

# Chapter 6

# Characterisation of a novel Gyrovirus product Human Apoptin

## *6.1 Introduction*

Chicken Anaemia Virus (CAV), first reported in 1979 in Japan, was the first gyrovirus to be discovered and studied extensively (Yuasa et al., 1979). In the years since that first discovery, 3 other gyroviruses have been reported, the Avian gyrovirus 2 (Rijsewijk et al., 2011), Human gyrovirus (Sauvage et al., 2011) and Human gyrovirus 3 (Phan et al., 2012).

Of interest to this study was the Human gyrovirus reported by Sauvage in 2011, this virus was shown to produce a protein that was similar in structure to Apoptin derived from CAV (Sauvage et al., 2011). It was then hypothesised that if this protein shared some sequence structure with Apoptin, then it could potentially share its tumour cytotoxicity and, possibly, be tumour specific. Intriguingly, this virus was discovered in Human tissue (Sauvage et al., 2011), and it could be assumed that the Human gyrovirus protein (referred to as Human gyrovirus Apoptin) could have a higher cytotoxic efficacy in Human tumour cells than Apoptin.

## *6.2 Results*

### 6.2 Analysis of Human gyrovirus Apoptin expression in relation to CAV Apoptin

To test the hypothesis that Human gyrovirus Apoptin behaves in a similar way to CAV Apoptin, Apoptin and Human gyrovirus Apoptin were compared in normal and transformed cell lines by Western blot analysis, Immunofluorescence and FACS analysis.

### 6.2.1 Generation of Human gyrovirus Apoptin

In order to begin the investigation of Human gyrovirus Apoptin, the protein needed to be generated from the sequence homology data reported (Sauvage et al., 2011). Alignment of CAV with HGyV revealed that there was a relatively low sequence identity overall, however, in the region of nucleotides 100-700, 70% identity was observed with a similar sequence organisation of the encoded proteins and the promoter region. The protein HGyV Apoptin was found to be comprised of 125 amino acids compared to 121 for CAV Apoptin with a low overall sequence similarity. In spite of this, HGyV Apoptin was shown to share the nuclear localisation signal, the nuclear export signal and phosphorylation sites with CAV Apoptin (Bullenkamp et al., 2012).

Synthetic HGyV Apoptin fused to GFP was generated by our group using primers overlapping by 15 bases. The resulting product was PCR amplified and the PCR product was cloned using TOPO TA (Invitrogen). The inserts were sequence verified and a correct HGyV-Apoptin gene (HGyV-AP) was removed from the TOPO vector and cloned in-frame with GFP into the mammalian expression vector pEGFP-C1 (Clontech) (Bullenkamp et al., 2012).

### 6.2.2 Expression of Human gyrovirus Apoptin and CAV Apoptin in colon cancer cells

Using the HCT116 colon cancer cell line, described in Chapter 2, expression of CAV Apoptin and Human gyrovirus (HGyV) Apoptin was studied. Cells were transfected with CAV Apoptin and HGyV Apoptin by Lipofectamine, described in Chapter 2. Western blot analysis was used to investigate the expression and molecular weight of HGyV Apoptin and compare the results to CAV Apoptin.

In agreement with the sequence, Western blot analysis showed HGyV Apoptin to be slightly larger than CAV Apoptin at 45kDa when fused to GFP, compared to 43kDa for CAV Apoptin fused to GFP (Figure 6.1).

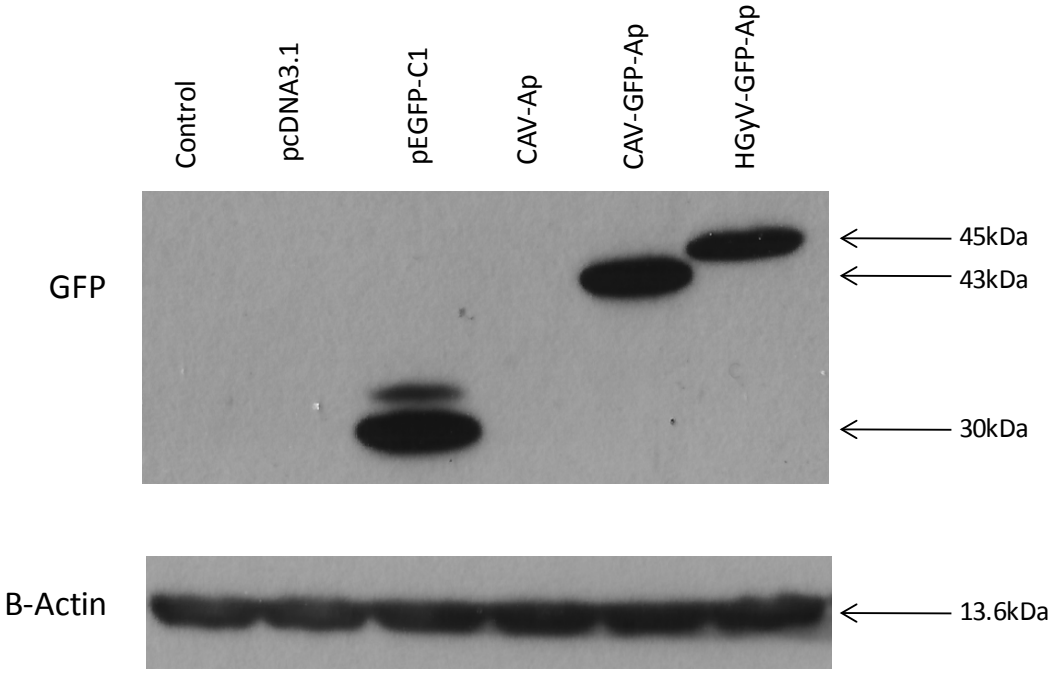


**Figure 6.1 Expression of Human Apoptin in HCT116 cells.** HCT116 cell line transfected with plasmids encoding GFP, CAV-Ap, GFP tagged CAV Apoptin and GFP-tagged Human Apoptin. Expression and molecular weight shown by Western blot analysis. Cells were grown for 72 hours.

### 6.2.3 Cellular distribution of CAV Apoptin and Human Apoptin in normal and transformed fibroblasts

Apoptin is a tumour specific protein and presents a distinct difference in localisation in normal and transformed cells. Human Apoptin has been reported to possess sequence similarity to CAV Apoptin and, as shown in Figure 6.1, the MW of the two proteins is very close. In order to investigate if these structural similarities extend to protein function, cellular distribution of the two proteins was compared by fluorescence microscopy.

Using primary fibroblasts, 1BR3, and their transformed isogenic match, 1BR3LT, expression and localisation of CAV and HGyV Apoptin was investigated by fluorescence imaging. Localisation analysis shows that the two proteins distribute in a similar manner in normal and transformed cells (Figure 6.2). In the normal cells both

GFP-tagged proteins localise in the cytoplasm and seem not to translocate into the nucleus. In addition, both GFP-tagged Apoptin proteins appear to cluster to form large aggregates in the cytoplasm of the normal cells which has been reported to be a precursor step in the degradation and removal of Apoptin in normal cell lines (Zhang et al., 2003). However, in transformed cells, a marked difference is observed with both CAV Apoptin and Human Apoptin localising to the nucleus of the cells (Figure 6.2)

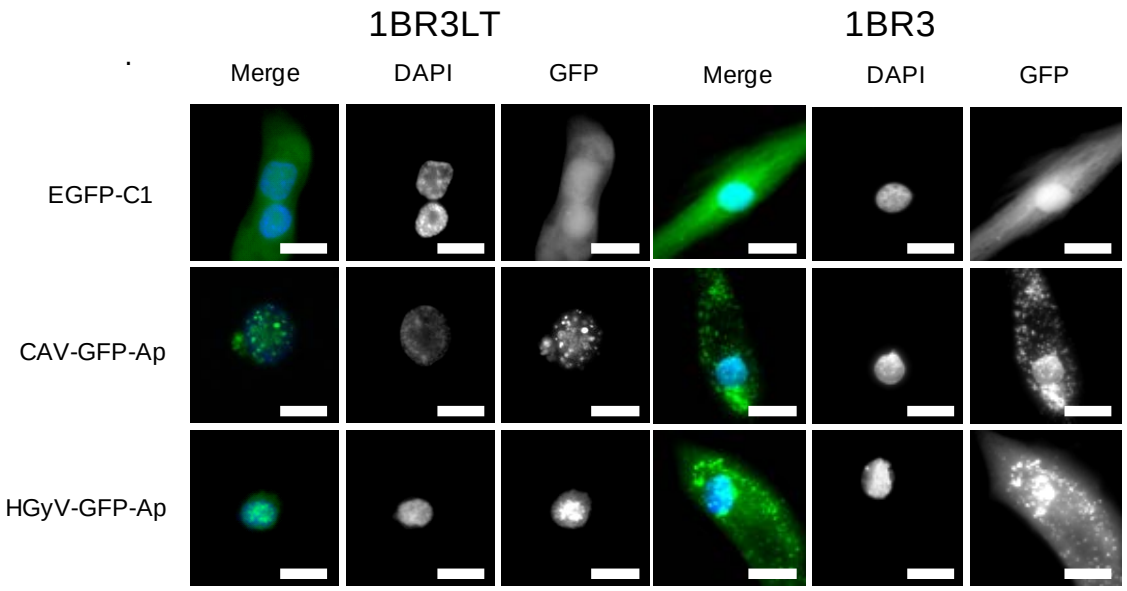


**Figure 6.2 Fluorescence images of localisation of Apoptin and Human Apoptin constructs in fibroblast cells.** Fluorescence imaging of 1BR3 and 1BR3LT transfected with GFP-tagged Apoptin constructs using Nucleofection. Slides were fixed and stained for DAPI. Cells were grown for 72 hours. 60x magnification. Scale bar: 25μm.

### 6.2.4 Cytotoxicity of Human Apoptin in comparison to CAV Apoptin

The results shown in Figure 6.2 indicate that HGyV Apoptin had very similar characteristics to CAV Apoptin when studying localisation in normal and transformed cell lines. To determine whether this specific localisation would result in transformed cell specific cytotoxicity, apoptosis was quantified by counting apoptotic nuclei in 1BR3 and 1BR3LT expressing HGyV Apoptin and CAV Apoptin.

Figure 6.3 shows that 1BR3 cells are resistant to CAV Apoptin, with no evidence of increase in killing but, HGyV Apoptin treatment induced 10% of cells to undergo apoptosis. However, in 1BR3LT (Figure 6.3), approximately 90% showed

killing with CAV Apoptin treatment decreasing slightly to approximately 80% killing when treated with HGyV Apoptin.

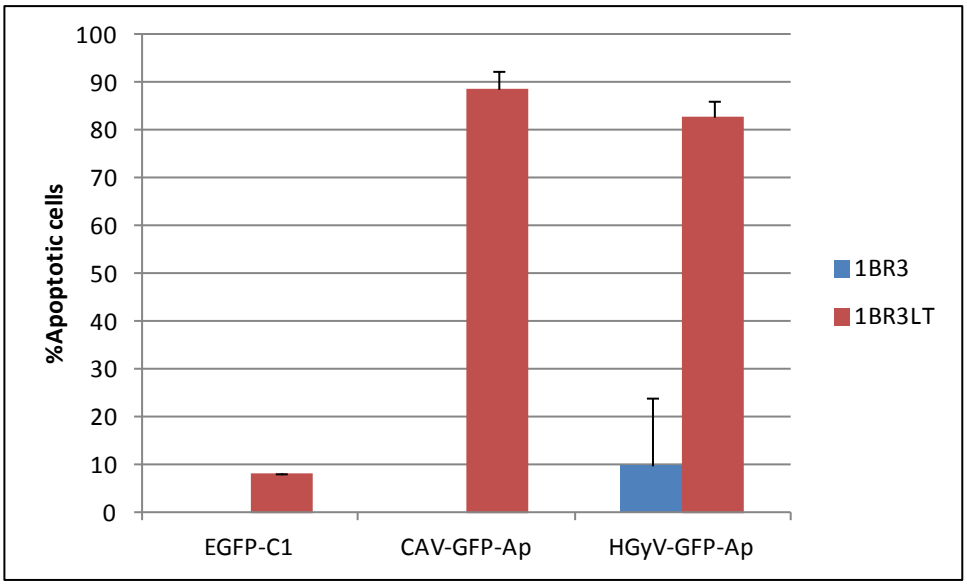


**Figure 6.3 Quantification of cell death in fibroblast cells treated with Apoptin and Human Apoptin.** Quantification of apoptotic cells by counting fragmented nuclei in 1BR3 and 1BR3LT cell lines transfected with Apoptin constructs. Error bars indicate standard deviation. Cells were grown for 72 hours. n=3. Data is specific to GFP positive cells only.

Further apoptosis analysis was performed to confirm this data using FACS analysis, which is less subjective, to investigate the effects of CAV Apoptin and HGyV Apoptin in these cell lines. As shown in Figure 6.4, 1BR3 cells treated with both viral proteins did not show a discernible change in cellular viability. Meanwhile, in 1BR3LT both CAV Apoptin and HGyV Apoptin significantly reduced cell survival, with CAV Apoptin killing approximately 18% of cells and HGyV Apoptin approximately 25% of cells. The reason for the discrepancies in the data between Figure 6.3 and 6.4 is because the results recorded in Figure 6.3 are representative of only the GFP positive cells, in Figure 6.4 all cells are recorded irrespective of GFP expression. This suggests an approximately 20% transfection efficiency in these cell lines.

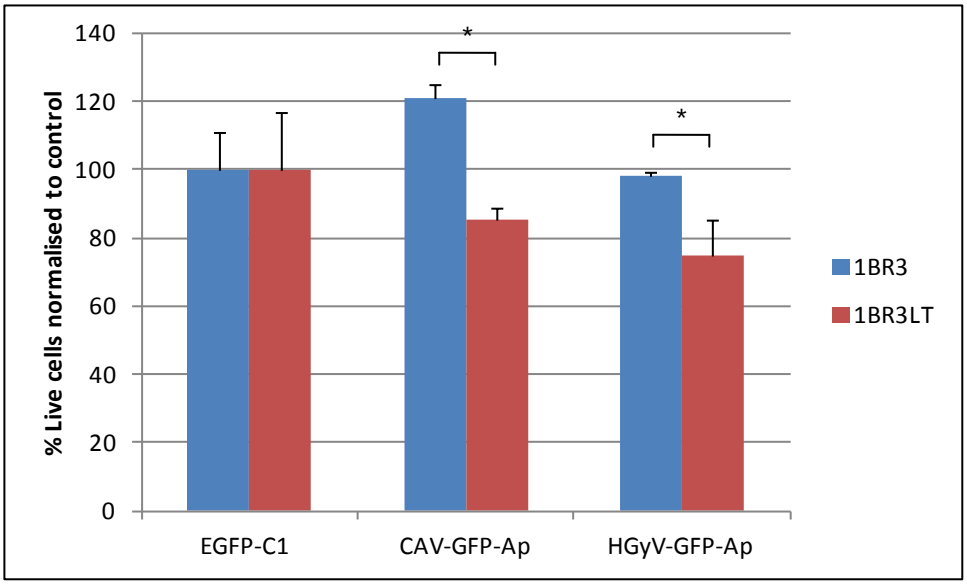


**Figure 6.4 FACS analysis of cell survival in fibroblast cell lines treated with Apoptin constructs.** Error bars indicate standard deviation. Cells were grown for 72 hours. n=3. Cell death shown here is from the total population of cells treated; this data does not take into account efficiency of adenoviral infection. For example, cells were not gated for Apoptin or GFP positive expression.

## *6.3 Discussion and Conclusions*

The discovery of a new protein with sequence homology to Apoptin is very exciting, and to find such a protein derived from a virus that was identified in human tissue makes the discovery even more intriguing. The purpose of this study was to compare the properties of HGyV Apoptin and CAV Apoptin to determine tumour specificity and potential cytotoxic efficacy in human cells.

Using a combination of techniques, HGyV Apoptin and CAV Apoptin were compared for their molecular weight, cellular distribution, cytotoxic effect and transformed cell specificity. Western blot analysis comparing protein size between CAV and HGyV Apoptin found that the two proteins were comparable in size (Figure 6.1).

This result was expected as sequence analysis of HGyV Apoptin shows similarities to CAV Apoptin (Sauvage et al., 2011).

Further investigation in normal 1BR3 and transformed 1BR3LT cell lines showed that the cellular distribution of HGyV Apoptin was very similar to CAV Apoptin, and in particular, the tumour specific localisation of CAV Apoptin was also shown using HGyV Apoptin (Figure 6.2). This specific localisation also related to increased toxicity and transformed cells expressing either form of Apoptin in the nucleus were found to be more apoptotic (Figure 6.3), suggesting that HGyV Apoptin has similar function to CAV Apoptin that have been previously reported (Danen-Van Oorschot et al., 2003; Tavassoli et al., 2005). This result provides evidence that HGyV Apoptin behaves in a similar way in normal and transformed cells as CAV Apoptin in these types of cells.

FACS analysis (Figure 6.4) confirmed the previous results and shows that HGyV Apoptin induces tumour specific apoptosis and is less toxic to normal cells, as shown with CAV Apoptin (Tavassoli et al., 2005).

Taken together, these data show that Human gyroviral Apoptin has a similar tumour specific cellular distribution pattern to CAV Apoptin, and the similarities extend to apoptotic potential in tumour cells. Human gyrovirus has yet to be linked to human disease, as was the case with CAV in chickens, so further study would be required to understand any potentially damaging properties the virus may have.

As has been shown using CAV Apoptin, kinase interaction in HGyV Apoptin is another important area of investigation, similar apoptotic and localisation properties may convey similar phosphorylation characteristics. Using kinases of interest from CAV Apoptin studies (Jiang et al., 2010a; Maddika et al., 2005; Maddika et al., 2009) it would be important to investigate the same kinases with HGyV Apoptin, potentially revealing tumour specific kinases linked to the apoptotic ability of each protein.

# Chapter 7
# General Discussion and Conclusion

## *7.1 Introduction*

The tumour specific characteristics of Apoptin have been shown to be linked to phosphorylation at a specific residue, T108. However, the specific kinase responsible for this phosphorylation in tumour cells is currently unknown. The aim of this study was to investigate potential kinases involved in Apoptin's T108 phosphorylation and subsequent apoptosis induction in tumour cells. The data presented in this study describes a tentative link between PKCβ1 and PKG-I expression and Apoptin function.

The two kinases of interest, PKG-I and PKCβ1, are expressed at differing levels correlating with the transformed status of a cell. For example, a transformed cell line expresses higher relative levels of PKCβ1 and lower relative levels of PKG-I, an observation that is reversed in normal cell lines. This trend is interesting because it is a clear differentiator between normal and transformed cells, at least those cell line models used in this study. In addition, Apoptin behaves in drastically different ways depending on the transformed status of the expressing cell.

Taking these observations together, a hypothesis was formed describing a potential link between the expression pattern of PKG-I and PKCβ1 and the function of Apoptin. The following pages describe the evidence for and the criticisms against such a hypothesis and my conclusions based on the data obtained.

## *7.2 Relevance of the kinase siRNA library*

Apoptin cytotoxicity has been shown to be linked to the phosphorylation status of the protein (Danen-Van Oorschot et al., 1997; Rohn et al., 2002), however, a kinase responsible has yet to be determined. The phosphorylation site of noted importance is threonine 108 (T108), requiring a serine/threonine kinase to phosphorylate its residue.

The siRNA library generated results that suggest that the kinases targeted were not relevant to Apoptin induced cell death (Figure 3.8). The siRNA library used targeted the p38 MAPK signalling pathway family of kinases which has been implicated in a range of responses from apoptosis to cell cycle checkpointing, differentiation, survival and transformation (Whitmarsh and Davis, 1996). This pathway encompasses a wide range of functions within the cell, so knockdown of members of this pathway would have non-specific effects resulting in observations such as reduced viability, seen in Figure 3.8. Further investigation of an Apoptin specific kinase would therefore need to be more specific and controlled to allow identification of true targets.

In conclusion, this particular section of the work succeeded in eliminating the 54 serine/threonine kinases targeted by this library from future investigation of the Apoptin tumour specific kinase.

## *7.3 PKC and PKG expression correlates with Apoptin sensitivity*

Data previously reported from this laboratory had shown that PKC isoforms were involved in the sensitivity of multiple myeloma (MM) cell lines to Apoptin and played a role in its function through phosphorylation (Jiang et al., 2010a). In particular, PKCβ1 phosphorylated Apoptin and, in return, was phosphorylated by Apoptin, resulting in Apoptin induced cell death in MM cells (Jiang et al., 2010a). This study also revealed that PKCβ1 expression was increased in Apoptin-sensitive, more malignant cells, suggesting that there could be a potential role for this kinase in Apoptin's tumour specificity.

In contrast, studies have shown that PKG expression is increased in normal cells and appears to be lost once cells become transformed (Hou et al., 2006). In

addition, *in silico* kinase prediction found that PKG was a potential kinase that interacts with Apoptin (Figure 3.9).

Normal and tumour cells were investigated for the expression pattern of these two kinases supporting the above findings. Apoptin sensitive tumour and transformed cells express high relative levels of PKCβ1 and much lower relative levels of PKG-I. Normal cells were found to possess much higher relative levels of PKG-I and a reduction in PKCβ1 expression levels, supporting evidence that suggests that PKCβ1 may have a tumour-specific role in Apoptin function. Initial results support the hypothesis that PKG-I expression could play an opposing role in Apoptin function, and may be involved in the protection of normal cells from Apoptin induced cytotoxicity.

## *7.4 PKC and PKG expression has limited effects on Apoptin function*

IP studies reveal that there is some physical interaction between PKG-I, PKCβ1 and Apoptin; however these findings are not conclusive evidence for a functional effect of such an interaction. Additionally, the IP studies conducted here used exogenously expressed proteins and therefore were not completely indicative of the environment within the cell line models used. Although some physical interaction is evident, it would have been very interesting to have tested the functional effects of these interactions by investigating the phosphorylation status of Apoptin pulled down by PKG-I, PKCβ1 and the two kinases together. It can be concluded that there is a trend that shows that physical interaction may occur between Apoptin and the two kinases of interest; however, owing to the inconclusive nature of the data and the technical issues presented, no definitive statement can be made in support of the hypothesis. Further work would be required to confirm or deny the existence of functional effects on Apoptin by PKG-I and PKCβ1 physical interaction.

The modification of kinase expression produced a variety of effects on Apoptin function in individual cell lines. For example, cells that were once resistant to Apoptin induced cytotoxicity (normal cells) became marginally more sensitive after overexpression of PKCβ1 or knockdown PKG-I expression. In the case of transformed cells, results were less convincing; knockdown of PKCβ1 was not successful enough to produce a significant change in kinase expression. Future work would require a more efficient method of silencing PKCβ1 expression, such as the method used in our

previous study in order to conclusively investigate the effect of PKCβ1 expression on Apoptin induced cytotoxicity (Jiang et al., 2010a). At the time of this study, however, this method was proving to be inconsistent. PKG-I overexpression was successful, but caused little effect in transformed cells aside from inducing some toxicity which has been observed in other studies (Fallahian et al., 2012; Fallahian et al., 2011). The data here suggests that for Apoptin cytotoxicity, the kinases PKG-I and PKCβ1 may be important in normal cells, however, based on cytotoxicity alone, this correlation is not observed in transformed cells. Additionally there was little correlation between kinase expression and phosphorylation of Apoptin in the cell lines tested, where a decrease in phosphorylation of Apoptin was most often accompanied by a decrease in total Apoptin expression, or vice versa, suggesting that kinase expression more likely affects the expression or stability of Apoptin. This could be further investigated by studying the stability and degradation of Apoptin by proteasomal inhibition experiments in combination with the kinase overexpression and knockdown techniques used here.

Apoptin localisation experiments performed here show that PKG-I and PKCβ1 kinase expression have some effect on the cellular distribution of the Apoptin protein. In normal cells the greatest effect on localisation is observed with a clear change from cytoplasmic to nuclear expression when PKCβ1 levels are higher and PKG-I levels are lower than basal. This set of observations does suggest that these kinases and their expression have some effect on Apoptin localisation; however, these experiments do not reveal the nature with which this distribution is affected. Further study using truncated and mutated variants of Apoptin would help to ascertain the mechanisms by which Apoptin localisation is affected. In transformed cells, the knockdown of PKCβ1, which has previously been shown to be ineffective, results in a reduced number of cells expressing Apoptin. These cells however still express Apoptin in the nucleus, suggesting that in this case the expression of Apoptin was reduced by kinase expression or siRNA treatment rather than there being any effects on Apoptin function and cellular distribution.

## *7.5 Human Apoptin, a promising tumour killing protein*

A new gyrovirus, recently discovered in human skin, was shown to produce a protein with sequence homology to Apoptin, referred to here as HGyV Apoptin (Sauvage et al., 2011). Using a combination of techniques it was shown that HGyV and

CAV Apoptin share tumour selective characteristics, in particular, cellular localisation and cytotoxicity. This set of data shows that HGyV Apoptin has a tumour specific cytotoxic potential comparable to CAV Apoptin, suggesting that with further investigation HGyV Apoptin could also be a potential targeted cancer therapy. It would also be very interesting to test the kinases studied here in the context of HGyV Apoptin. This kind of study could not only help to reveal the specific functional effects that PKCβ1 and PKG-I have on HGyV Apoptin, but could also provide further information about the function of CAV Apoptin.

## *7.6 Conclusion*

To summarise, the data presented here, although inconclusive, does indicate that Apoptin function is affected by the expression of the protein kinases PKG-I and PKCβ1. Differential expression of these kinases has been shown to correlate with the transformed phenotype of the cell lines studied and it is widely accepted that Apoptin behaves differently in normal and transformed cell lines. The aim of this work was to determine whether kinase expression had any involvement in Apoptin function or whether differential kinase expression was just a consequence of the transformed phenotype and had no bearing on the mechanism of Apoptin cytotoxicity.

Cytotoxicity assays found that there was a trend towards PKG-I expression and Apoptin resistance and PKCβ1 expression and Apoptin sensitivity, suggesting that cells with an expression pattern fitting the observations made here could have a predictable response to Apoptin treatment. For example, those cells with a higher relative PKG-I expression level and reduced PKCβ1 expression could be predicted to be resistant to Apoptin. As this study did not conclusively show that the kinase expression directly affected Apoptin function it can only be concluded that modification of PKG-I and PKCβ1 expression creates an environment within the cell that causes changes in Apoptin behaviour. The effects on the cell could be to induce a more or less transformed phenotype which would in turn change the function of Apoptin and give rise to the observations seen here. Interestingly, changes in the kinase expression in the cells studied induced a change in the response to cisplatin treatment. This is an indication that PKG-I and PKCβ1 kinase expression may affect sensitivity to apoptosis inducing agents rather than Apoptin specifically. Cisplatin is a tumour specific drug so this finding also supports phenotypic change (cells becoming more or less transformed)

as a consequence of kinase expression modification. Phosphorylation experiments were inconclusive and so a connection with kinase expression was not clearly shown. The effects of kinase expression appeared to be to inhibit or increase the expression of Apoptin. Localisation results also show that Apoptin expression and distribution is in some way affected by the expression of PKG-I and PKCβ1, however, this again could be explained by a change in the phenotype of the cells rather than specific interaction or direct effects of kinase expression on Apoptin function. Increased nuclear expression of Apoptin is associated with increased PKCβ1 and decreased PKG-I expression. However, as mentioned previously, these results do not clearly show that this is a direct consequence of the kinases themselves and their interaction with Apoptin or phenotypic changes induced by kinase expression which result in differing distributions of the Apoptin protein.

The results presented in this study are useful because they highlight the different functions of Apoptin depending on the transformed nature of a cell. Apoptin is cytotoxic in many transformed cell types and relatively harmless in normal counterpart cells, and the mechanisms by which this specificity is determined are still largely unknown. Phosphorylation is thought to be a key step in specificity and this study shows that there is some evidence for phosphorylation determined by PKG-I and PKCβ1. Apoptin localisation is also important, and again, a link between the kinases studied and Apoptin localisation is shown.

A trend towards Apoptin function and PKG-I and PKCβ1 expression could be implied from this research, and previous work has already shown a link between PKCβ1 and Apoptin function (Jiang et al., 2010a). Further, the evidence presented here for the regulation of PKCβ1 expression by PKG-I is intriguing, rather than a direct effect of PKG-I on Apoptin, this kinase could in fact reduce the expression of PKCβ1 thus impairing the function of Apoptin.

On reflection, although the work presented is not entirely conclusive, the evidence does suggest that there is a link between the expression of PKG-I and PKCβ1 and the function of Apoptin. Whether this link is a result of direct interaction, such as phosphorylation or physical modification, or even the changes in the cell associated with kinase expression, is not clear, however, this study highlights a novel approach to the investigation of Apoptin function and a new avenue for further study of this complex protein.

Additionally, the novel discovery of HGyV Apoptin, and the potential discoveries that could be made through further investigation of this viral protein in concert with CAV Apoptin, could prove very exciting for the field of targeted cancer therapies.

## *7.7 Future Perspectives*

Cancer is still a major killer in the Western world and many therapeutic strategies have been developed to target the specific biological differences inherent in cancers derived from different tissues. However, there is still hope for the management of this disease. Many properties of transformed cells are shared between different cancers, even if the specific causes are different. For example, the Hallmarks of cancer, described in Chapter 1, highlights common characteristics observed in all forms of cancer, from increased, uncontrolled proliferation to evasion of the body's own methods of cell growth control. In addition, all cancer cells overexpress and underexpress certain proteins and kinases compared to their normal counterparts, and the results reported here suggest that Apoptin may exploit these differences in a variety of tumour cells, being specific to cancer rather than being specific to certain types of cancer.

In the future, the advantages of an agent, such as Apoptin, that could be used to treat a variety of tumour types, would be a major aid in the management of cancer, potentially obviating the need for individual therapies for different forms of the disease.

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# Appendix

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*Therapeutics, Targets, and Chemical Biology*

Cancer Research

## Crucial Roles for Protein Kinase C Isoforms in Tumor-Specific Killing by Apoptin

Jie Jiang[1], Daryl Cole[2], Nigel Westwood[1], Lee Macpherson[1], Farzin Farzaneh[1], Ghulam Mufti[1], Mahvash Tavassoli[2], and Joop Gäken[1]

### Abstract

The chicken anemia virus–derived protein apoptin induces apoptosis in a variety of human malignant and transformed cells but not in normal cells. However, the mechanisms through which apoptin achieves its selective killing effects are not well understood. We developed a lentiviral vector encoding a green fluorescent protein–apoptin fusion gene (LV-GFP-AP) that can efficiently deliver apoptin into hematopoietic cells. Apoptin selectively killed the human multiple myeloma cell lines MM1.R and MM1.S, and the leukemia cell lines K562, HL60, U937, KG1, and NB4. In contrast, normal $CD34^+$ cells were not killed and maintained their differentiation potential in multilineage colony formation assays. In addition, dexamethasone-resistant MM1.R cells were found to be more susceptible to apoptin-induced cell death than the parental matched MM1.S cells. Death susceptibility correlated with increased phosphorylation and activation of the apoptin protein in MM1.R cells. Expression array profiling identified differential kinase profiles between MM1.R and MM1.S cells. Among these kinases, protein kinase Cβ (PKCβ) was found by immunoprecipitation and *in vitro* kinase studies to be a candidate kinase responsible for apoptin phosphorylation. Indeed, shRNA knockdown or drug-mediated inhibition of PKCβ significantly reduced apoptin phosphorylation. Furthermore, apoptin-mediated cell death proceeded through the upregulation of PKCβ, activation of caspase-9/3, cleavage of the PKCδ catalytic domain, and downregulation of the MERTK and AKT kinases. Collectively, these results elucidate a novel pathway for apoptin activation involving PKCβ and PKCδ. Further, they highlight the potential of apoptin and its cellular regulators to purge bone marrow used in autologous transplantation for multiple myeloma. *Cancer Res; 70(18); 7242–52. ©2010 AACR.*

### Introduction

Stem cell rescue following high-dose cytotoxic chemotherapy is considered curative and has gained extensive application worldwide as a therapeutic modality in several hematologic malignancies (1–4). Allogeneic bone marrow transplant is the preferred choice for most types of leukemias, but due to the rarity of HLA-compatible donors, autologous bone marrow transplantation is still an alternative therapeutic option. However, disease relapse remains a primary cause of death, partially due to inefficient elimination of contaminated clonogenic tumor cells from the autografts. Multiple pharmacologic and immunologic approaches aiming at the elimination of leukemic cells have been developed; however, to date, these purging methods have shown only limited efficacy, restricting their clinical applications (5, 6). In addition, several gene therapy–based trials have been conducted for the selective removal of contaminating epithelial cancer cells from autografts. However, these strategies have been shown to be inefficient for purging of leukemia cells mainly due to the lack of efficient gene delivery into hematologic malignant cells (7, 8).

Apoptin, a chicken anemia virus–derived protein, has been shown to possess tumor-specific cytotoxicity (9, 10). Its expression induces apoptosis in human tumor and transformed cells, but there is little or no cytotoxic effect in many normal human cell lines derived from different tissues, including peripheral blood mononuclear cells, fibroblasts, and epithelial cells (11–13). Several studies have shown that the tumor-specific killing of apoptin correlates with its phosphorylation and its subcellular localization (14, 15). In cancer cells, apoptin is localized in the nucleus and is phosphorylated on Thr-108 by an as yet unknown kinase (16, 17), whereas in normal cells apoptin is detected in the cytoplasm and is essentially unphosphorylated. Recent studies have reported that apoptin interacts with the p85 Src homology 3 (SH3) domain of phosphatidylinositol 3′-kinase (PI3K) and protein kinase B (AKT) in MCF7 breast and PC3 prostate cancer cell lines. The activation of the PI3K/AKT pathway by apoptin results in the induction of the cyclin-dependent kinase CDK2, in turn leading to the phosphorylation of apoptin (18, 19).

**Authors' Affiliations:** [1]Department of Haematological and Molecular Medicine, The Rayne Institute, King's College London and [2]Head and Neck Oncology Group, Department of Oral Pathology, King's College London Dental Institute, Guy's Hospital Campus, London, United Kingdom

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

M. Tavassoli and J. Gäken authors contributed equally.

**Corresponding Author:** Mahvash Tavassoli, Head and Neck Oncology Group, Department of Oral Pathology, King's College London Dental Institute, Guy's Hospital Campus, London SE1 9RT, United Kingdom. Phone: 44-207-1888321; Fax: 44-207-1884375; E-mail: mahvash.tavassoli@kcl.ac.uk.

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In contrast, we have found no correlation between AKT activity in human cancer cell lines and their sensitivities to apoptin killing.[3]

In this study, we used a matched pair of multiple myeloma cell lines (MM1.R and MM1.S) that show resistance (R) or sensitivity (S) to dexamethasone, with MM1.R being derived from MM1.S through continuous growth in dexamethasone. These cell lines showed a marked difference in sensitivity to apoptin-induced killing, with MM1.R being clearly more sensitive than MM1.S. Microarray expression profiling of these cell lines showed that several kinases, including members of the protein kinase C (PKC) family, are overexpressed in the apoptin-sensitive cell line MM1.R compared with MM1.S cells (see Supplementary Table S1).

PKC is a family of serine-threonine kinases, composed of at least 13 known isoforms with a wide range of tissue distribution, subcellular localization, and function. All PKC isoforms contain a highly conserved COOH-terminal catalytic domain and $NH_2$-terminal regulatory domain with an autoinhibitory pseudosubstrate sequence (20–22). It has been well established that the activation of the classic PKC isoforms (α, β1, β2, and γ) requires $Ca^{2+}$ and a phospholipid, such as diacylglycerol (DAG) or phosphatidylserine (PS). These PKC isoforms also contain two cysteine-rich motifs within their $NH_2$-terminal regulatory domain that facilitate phorbol ester (TPA) interaction and subsequent activation. The novel isoforms (δ, ε, η, μ, θ) are also activated by DAG and phorbol esters; however, they lack a complete C2 domain responsible for $Ca^{2+}$ interaction and are therefore $Ca^{2+}$ independent. The protein structures of atypical PKC isoforms (ζ, ι/λ) differ from the other members of the PKC family, missing both binding regions for $Ca^{2+}$ and DAG or phorbol esters. Activation of atypical isoforms is dependent on other phospholipids, such as PS, inositol lipids, and phosphatidic acid.

Most PKC isoforms are present in the cytosol in nonstimulated cells. Upon stimulation, several PKC isoforms migrate to the plasma membrane where interaction with DAG induces full activation. A number of activated PKC isoforms can also translocate to the nucleus where they can be targeted by lipid coactivators or other activated protein kinases (23). Furthermore, some PKC isoforms such as PKCδ can be activated by caspase-3 cleavage to release an active catalytic domain from the inhibitory $NH_2$-terminal regulatory domain.

Increased level of PKC or differential activation of PKC isoforms has been linked to a variety of cancers, including breast, lung, thyroid, and adenomatous pituitary cancers as well as leukemias (24, 25). There is emerging evidence that PKCs play key roles in the regulation of cell growth, apoptosis, and differentiation of hematopoietic cells. Studies involving small interfering RNA knockdown and genetic disruption of individual PKC isoforms in mice have shown that PKCα, β, λ, ε, and ζ preferentially function to promote cell proliferation and survival, whereas PKCδ is a critical proapoptotic kinase in many cell types (26).

[3] Unpublished data.

To evaluate apoptin tumor-specific toxicity in hematologic malignancies, we developed a lentiviral vector encoding a green fluorescent protein–apoptin fusion gene (LV-GFP-AP) that can be efficiently delivered into hematopoietic cells. This strategy enabled us to identify novel apoptin-interacting cellular targets. Here, we provide strong evidence for the role of PKC family kinases, particularly PKCβ variants, in apoptin phosphorylation and consequently its tumor-specific cytotoxicity.

## Materials and Methods

Additional details about the materials and methods can be found in the Supplementary Data.

### Cell lines

MM1.S (dexamethasone-sensitive) and MM1.R (dexamethasone-resistant) cell lines were obtained from Dr. Tai (Dana-Farber, Boston, MA). K562 (*BCR/ABL* cytogenetics), HL60 (TPA, retinoic acid differentiation), U937 (TPA differentiation), and KG1 (monosomy 7, trisomy 8 cytogenetics) leukemic cells were obtained from the American Type Culture Collection. NB4 (t15,17 translocation) was obtained from German Collection of Microorganisms and Cell Cultures. HCT116 (*KRAS* mutation analysis) was obtained from Prof. Vogelstein (Johns Hopkins, Baltimore, MD). All cell lines were obtained since 2006 and were tested for cell line–specific defects before batch freezing (see above for specific assays). Cells are regularly tested to ensure the absence of *Mycoplasma* contamination, and cell morphology is regularly checked to ensure the absence of cross-contamination of cell lines.

### Construction of lentiviral vector LV-GFP-AP, lentivirus production, and titration

LV-GFP-AP was constructed by cloning GFP-apoptin derived from pCMV-GFP-Ap (13) into the LV-GFP lentiviral construct (27) through replacement of GFP by GFP-AP by blunt end ligation (see Supplementary Fig. S1A). Lentiviral vectors were produced by cotransfection of 293T cells with the second-generation packaging plasmid pCMVΔ8.91 and plasmid pMDG encoding VSV-G–pseudotyped envelope (27). The titer of lentivirus was determined by quantification of viral core protein p24 through enzyme-linked immunosorbent assay (ELISA) using a HIV-1 p24 capture assay kit (Perkin-Elmer). The values were normalized against a recombinant p24 protein standard. Virus numbers were then calculated based on the fact that a viral particle contains 2,000 p24 molecules (28, 29). Comparable virus titers, based on p24 ELISA, were achieved for LV-GFP and LV-GFP-AP (Supplementary Table S2).

### MTT proliferation

Leukemia cell lines were infected with LV-GFP or LV-GFP-AP in the presence of 4 μg/mL polybrene. Cells were seeded at $5 \times 10^4$, $10^4$, and $10^3$ per well in 96-well plates for MTT assays. On days 2, 4, 6, 8, 10, 12, and 14 postinfection, 20 μL of 5 mg/mL MTT in PBS were added to each well and samples were incubated for 2 to 4 hours. MTT solubilization solution (100 μL) was then added, and samples were incubated overnight. The optical density (OD) was measured, and the OD values

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were converted into percentages of the control absorbance. The average values were obtained from triplicates.

**Flow cytometric analysis**

Apoptosis was assessed by propidium iodide (PI) staining. Briefly, cells were pelleted and resuspended in PBS with 20 μg/mL of PI. The settings for fluorescence-activated cell sorting (FACS) analysis were based on unstained parental, GFP-transduced, and PI-stained control samples. The cells were analyzed on a BD FACSCanto II (Becton Dickinson), and 10,000 events were acquired per sample. Fluorescence data were analyzed by FlowJo software.

**Methylcellulose colony-forming assay**

Normal human bone marrow cells were obtained from volunteers undergoing open heart surgery at King's College Hospital (Ethics Committee number 05-03-125). $CD34^+$ cells were isolated using anti-$CD34^+$ microbeads, and an AutoMacs fractionation device (Miltenyi Biotec) according to the manufacturer's instructions. $CD34^+$ cells were cultured in Stemspan serum-free medium with cytokine cocktail (StemCell Technologies) for 48 hours. Mobilized $CD34^+$ cells were infected with LV-GFP or LV-GFP-AP at a multiplicity of infection (MOI) of 100. Two days postinfection, infected $CD34^+$ cells were analyzed for their progenitor function by an *in vitro* colony-forming assay (StemCell Technologies).

**Purification of maltose-binding protein–apoptin fusion protein**

Apoptin cDNA was cloned into pMALc2 (NEB) in frame with the maltose-binding protein (MBP), and protein was purified from BL21 codon plus *E. coli* cells (Stratagene) using an Amylose Resin kit (NEB) according to the manufacturer's recommendations.

**Immunoprecipitation and Western blotting**

For immunoprecipitation, cells were lysed in radioimmunoprecipitation assay buffer on ice with freshly added protease inhibitor cocktail (Sigma). The cell debris was removed, and 2 μg of anti-GFP (Abcam) or anti-PKCβ1 antibody (Santa Cruz Biotechnology) were added to the cell lysate for 30 minutes at room temperature under constant agitation. Twenty microliters of washed Bio-Adembeads PAG (Ademtech) were added to the cell lysate with antibody for 2 to 3 hours at 4°C, and complexes were pulled down using a magnet rack.

Proteins from different cell fractions were isolated using the ProteoJET cytoplasmic and nuclear protein extraction kit (Fermentas) according to the manufacturer's protocol.

For Western blotting, the proteins were separated on SDS-PAGE gels, electroblotted onto nitrocellulose membrane (GE Healthcare), and blocked with 5% nonfat dried milk for 1 hour at room temperature. The membrane was incubated with a specific primary antibody and a horseradish peroxidase–conjugated secondary antibody and developed using an enhanced chemiluminescent (ECL) detection system and exposed to ECL film (both from Amersham Biosciences). The blot was stripped with ReBlot plus strong antibody stripping buffer (Millipore) and reprobed with a different antibody as required.

Antibodies against Thr-108–phosphorylated apoptin or total apoptin were raised in rabbit against peptides H2N-SLITTT(PO3H2)PSRPRTA-CONH2 and H2N-SLITTTPSRPRTA-CONH2, respectively (Eurogentec). All antibodies used in the study were purchased from Santa Cruz Biotechnology except antibodies against MERTK, DGKH, and GFP for immunoprecipitation, which were purchased from Abcam. Antibodies against phospho-AKT-Ser-473, phospho-PKCβ2-Thr-638, total AKT, PI3K/p85, caspase-3, caspase-9, and GFP for Western blotting were purchased from Cell Signaling.

***In vitro* kinase assay**

Two micrograms of MBP-apoptin fusion protein were added to 1× kinase buffer (Cell Signaling) containing 200 μmol/L ATP and 0.2 ng of PKCβ2 kinase (Cell Signaling) or PKCβ1 pulled down from MM1.R cell lysate. The reaction mixture was incubated for 30 minutes at room temperature. Reactions were carried out under different conditions, with kinase or without kinase, with PKCβ-specific inhibitor (Calbiochem) or AKT-specific inhibitor (Calbiochem). The reaction was stopped by adding 2× loading buffer and boiling for 5 minutes.

**Construction of plasmids encoding PKCβ catalytic domains and transfection of 293T**

The catalytic domains of PKCβ1 and PKCβ2 were PCR amplified from cDNA derived from MM1.R cells and cloned into the retroviral vector pBabePuro. The forward primer introduces a start codon in a Kozak context. The sequences were verified, and the expression of the catalytic domain was verified by Western blotting of transfected cells with PKCβ1- and PKCβ2-specific antibodies (Santa Cruz Biotechnology).

**Immunohistochemistry and visualization by fluorescence microscopy**

HCT116-p53−/− or MM1.R cells ($5 \times 10^4$) were infected with a lentiviral vector. At day 2 or 5, cells were fixed, permeabilized, and incubated with antibody against PKCβ1 for 1 hour and Texas red IgG secondary antibody for 1 hour. The cells were washed and covered with 4′,6-diamidino-2-phenylindole mounting medium (Vector Laboratories) and visualized.

**Statistical analysis**

Student's *t* test was used to determine significance.

## Results

**Apoptin induces tumor-specific killing of leukemia cells**

A panel of leukemia cell lines as well as primary $CD34^+$ and PBMC cells were infected with LV-GFP or LV-GFP-AP lentiviral vectors. In general, the leukemia cell lines were found to be more sensitive to lentiviral infection than normal cells. In particular, multiple myeloma MM1.R and MM1.S cells were highly sensitive to infection, requiring only a MOI of 2 to achieve 100% transduction (Supplementary Fig. S2A; MM1.S cells have a comparable infectivity to the parental matched MM1.R cells; data not shown). $CD34^+$ cells were less sensitive, requiring a higher MOI of 100 to obtain 50% to 80% transduction efficiency. Furthermore, higher expression levels of

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the transgene were observed in the leukemic cell lines compared with primary $CD34^+$ cells or PBMCs (Supplementary Fig. S2B and C).

To examine apoptin-mediated cell death, $1 \times 10^5$ cells were infected with equal MOIs for LV-GFP and LV-GFP-AP virus. After 5 days, cell death was determined by FACS analysis of PI-positive cells. As shown in Fig. 1A, GFP-apoptin triggered apoptosis in transduced leukemia cells in a dose-dependent manner, whereas no cell death was observed in untransduced or GFP-transduced parental cells (Fig. 1A). Furthermore, MTT cell survival assay showed more than 90% cell death in a panel of leukemia cell lines infected with LV-GFP-AP, whereas no significant killing was observed with LV-GFP–infected and uninfected parental cells (Fig. 1B). Using a MOI of 100, we were

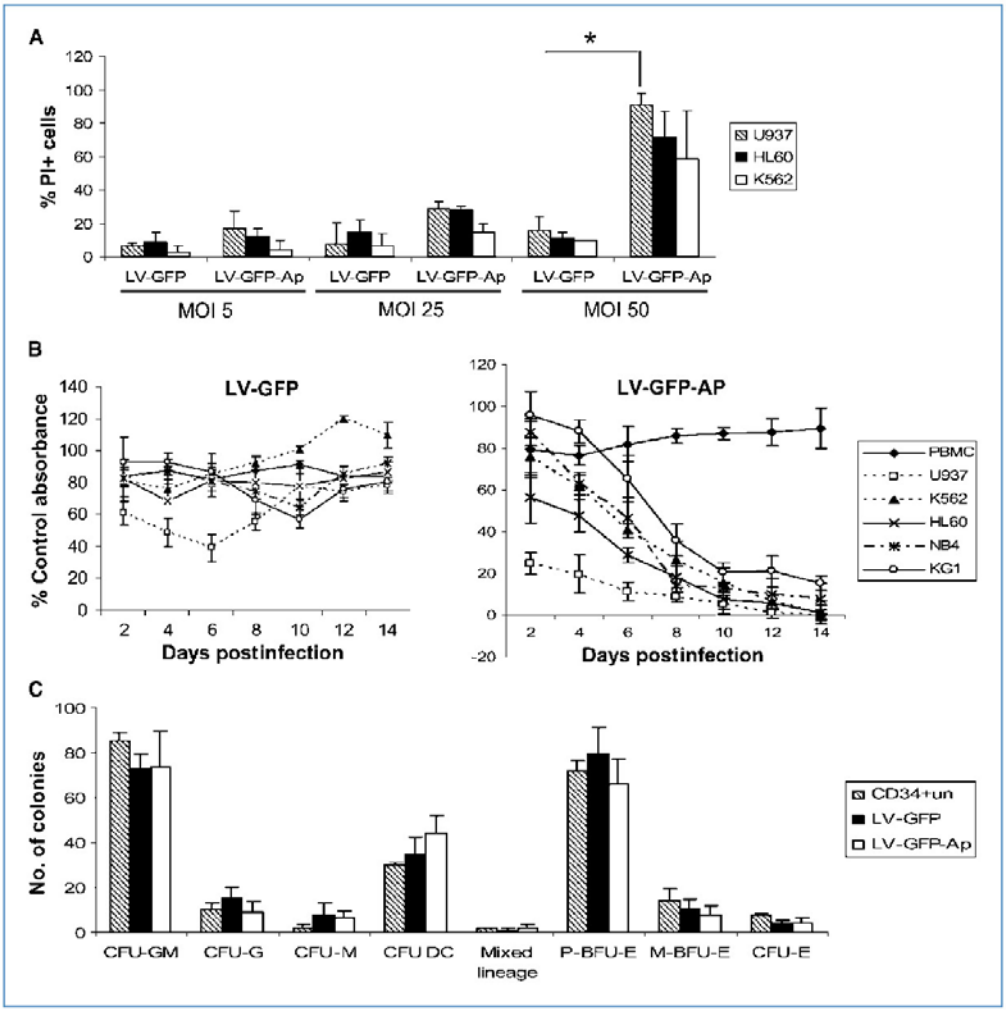


Figure 1. Apoptin kills leukemia cell lines but does not kill normal bone marrow $CD34^+$ cells. A, cell death was measured by FACS analysis of PI-positive cells on day 5 postinfection. The mean values were calculated from three independent experiments. *, $P < 0.05$. B, cell viability of MOI 100 infected cells was measured by MTT assay at the indicated time points. The values were converted to percentage of control-untransduced parental cells. The mean values and SD were obtained from three independent experiments each performed in triplicate. C, colony-forming assay of $CD34^+$ cells. Mobilized normal $CD34^+$ cells were infected with a MOI of 100 and 48 h later seeded in methylcellulose-based medium for the evaluation of colony formation. After 12 to 14 d, the different lineage colonies were counted and the mean values were obtained from two independent experiments each in duplicates.

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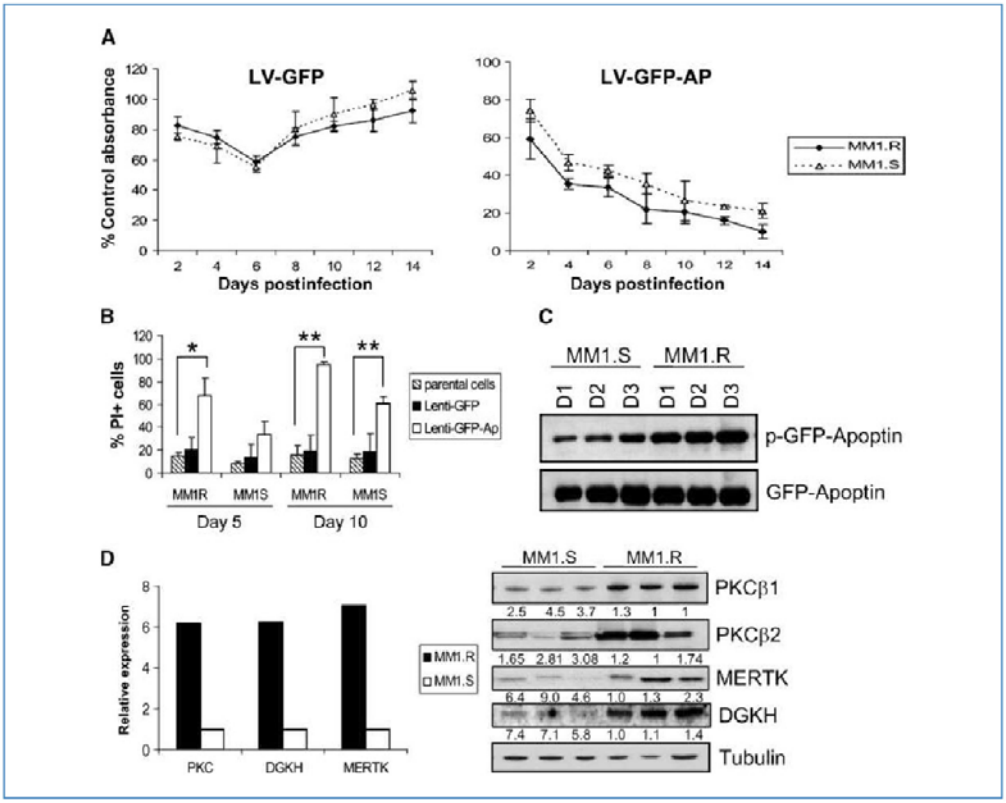


**Figure 2.** Sensitivity to apoptin killing is correlated to PKCβ expression in myeloma cells. A, cell viability was measured by MTT assay of MOI 2 infected cells at the indicated time points. The values were determined by the percentage of the value measured from control-untransduced parental cells. The mean values and SD were obtained from three independent experiments performed in triplicate. B, cell death was measured by FACS analysis of PI-positive cells on day 5 or day 10 of MOI 2 infected cells, respectively. The mean values were derived from three independent experiments. *, $P < 0.05$; **, $P < 0.01$. C, differential phosphorylation of apoptin protein was detected by Western blotting in MM.1R and MM1.S cells expressing GFP-apoptin. D1, D2, and D3 represent day 1, day 2, and day 3 postinfection. D, relative expression of PKCβ, DGKH, and MERTK in MM1.R and MM.1S cells measured by real-time quantitative RT-PCR (left) and their protein levels were measured by Western blotting (right). PKCβ primers were designed to recognize both PKCβ variants. PKCβ protein level was detected by variant-specific antibodies. Protein samples were prepared from three independent cell populations. Numbers below each lane represent the quantified level of protein as compared to the control.

able to infect more than 80% of $CD34^+$ cells with LV-GFP and more than 50% with LV-GFP-AP, respectively, but the transgene expression was 2 to 3 logs lower compared with the leukemia cells (Supplementary Fig. S2C). Nevertheless, after infection with LV-GFP-AP, $CD34^+$ cells remained viable and maintained the same differentiation capacity as control LV-GFP–infected and uninfected $CD34^+$ cells on methylcellulose colony-forming assay (Fig. 1C). This indicates that apoptin-expressing $CD34^+$ cells function normally as hematopoietic progenitors and can differentiate into multiple lineages. The low infectivity, low expression, and lack of toxicity shown by the transduced $CD34^+$ and PBMC cells show an important potential for LV-GFP-AP as a purging agent in autologous bone marrow transplant for leukemia.

### Identification of apoptin kinase(s) by microarray analysis

Two variants of multiple myeloma cells, which originated from the same patient, are characterized by resistance (MM1.R) or sensitivity (MM1.S) to dexamethasone. Using a MOI of 2, we showed that MM1.R cells were significantly more sensitive to apoptin-induced cell death as assessed by MTT cell viability and FACS analysis (Fig. 2A and B). Importantly, increased sensitivity of MM1.R cells to apoptin-induced cell death was associated with a higher level of apoptin phosphorylation (Fig. 2C) detected by Western blot analysis using an apoptin phospho-specific antibody that we have recently developed.

Expression profiling by Affymetrix microarray analysis showed the overexpression of several kinases in MM1.R cells

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compared with MM1.S cells, including PKCβ (9 fold), c-mer proto-oncogene tyrosine kinase (MERTK, 5.9-fold), and diacylglycerol kinase (DGKH, 5.2-fold). The expression levels of PKCα (1.6-fold), PKCγ (1.5-fold), and PKCδ (1.4-fold) were slightly higher in MM1.R cells. In contrast, PKCε expression was higher (3.2-fold) in MM1.S cells. The detailed microarray data are provided in the supplementary materials. The highest expressing candidates PKCβ, MERTK, and DGKH were further validated by quantitative reverse transcriptase-PCR (qRT-PCR) using Sybr Green Taqman master mix and Western blot analysis (Fig. 2D). These experiments confirmed the microarray analysis data.

### PKCβ interacts and phosphorylates apoptin *in vitro* and *in vivo*

To further investigate the role of the identified kinases in the phosphorylation of apoptin, MM1.R cells were infected with LV-GFP or LV-GFP-AP. After 3 days, total cell extracts were immunoprecipitated with GFP antibody (Abcam). Western blot analysis of the IP complexes detected a clear IP of both PKCβ variants with GFP-apoptin fusion protein but not with GFP alone. The precipitated complexes were also analyzed using antibodies against DGKH, MERTK, AKT, PI3K/p85, PKCα, and PKCδ, but none of these proteins were found in the GFP-apoptin immunocomplex (Fig. 3A). The reciprocal IP using anti-PKCβ1 (Fig. 3B) or PKCβ2 antibody confirmed the interaction of the PKCβ variants with GFP-apoptin.

To further investigate whether apoptin is phosphorylated by PKCβ, bacterial recombinant apoptin fused to MBP (MBP-apoptin) was used as substrate with either recombinant GST-PKCβ2 protein (Cell Signaling) or with immunopurified PKCβ1. Western blot analysis confirmed enhanced

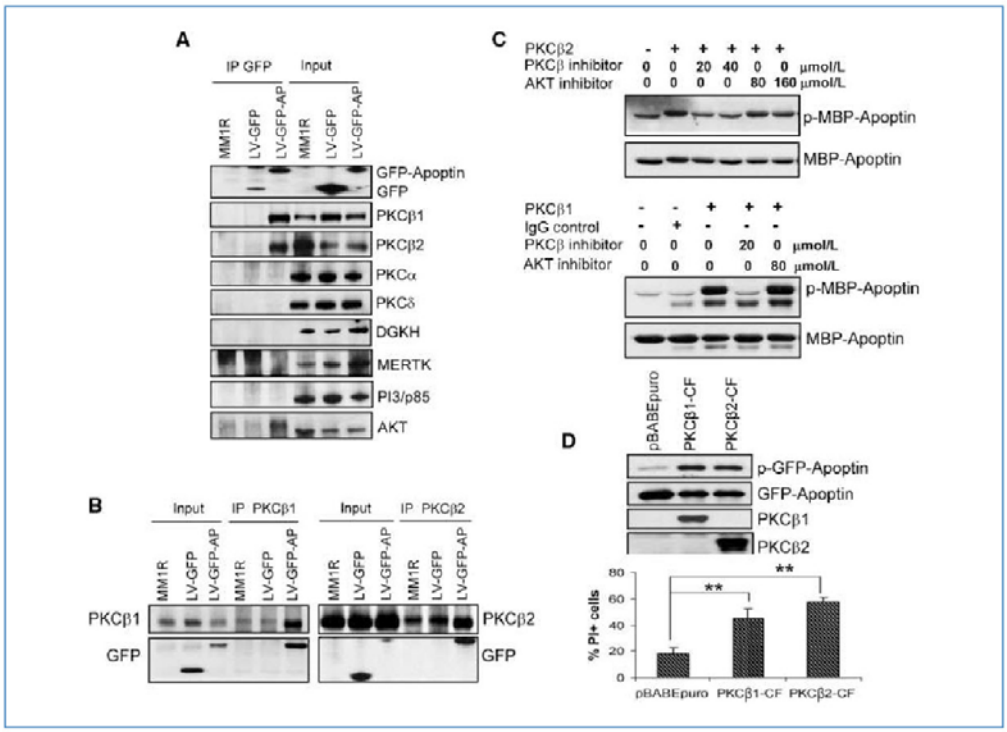


**Figure 3.** Both PKCβ variants interact with apoptin and phosphorylate apoptin *in vitro* and *in vivo*. MM1.R cells were lysed in RIPA buffer on day 3 postinfection. A, Immunoprecipitation (IP) with anti-GFP antibody. B, IP with anti-PKCβ1 antibody (left) and anti-PKCβ2 antibody (right). The immunocomplexes were separated on SDS-PAGE gels, and proteins were detected by the indicated antibodies. C, an *in vitro* kinase assay showed MBP-apoptin phosphorylation by recombinant PKCβ2 (top) and PKCβ1 purified from MM1.R cell lysates by IP with a PKCβ1 antibody or an unspecific antibody (lane 2, bottom). The lower bands are heavy chains from the IP cross-reacting with the secondary antibody. The phosphorylation of MBP-apoptin is blocked by a PKCβ inhibitor, but not by an Akt inhibitor. D, increased phosphorylation of apoptin was detected in 293T cells 48 h after cotransfection of LV-GFP-AP with pPKCβ1-CF and pPKCβ2-CF. Expression of PKCβ1 and PKCβ2 catalytic fragments in 293T cells was detected by Western blot using PKCβ1- and PKCβ2-specific antibodies. The same membranes were reprobed with a phosphorylated apoptin-specific antibody and anti-GFP antibody. Increased cell death was detected by FACS analysis in 293T cells 5 d after cotransfection of LV-GFP-AP with pPKCβ1-CF and pPKCβ2-CF. The mean values and SD were obtained from triplicate experiments. **, $P < 0.01$.

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phosphorylation of apoptin by both PKCβ1 and PKCβ2 (Fig. 3C). Furthermore, a PKCβ-specific inhibitor resulted in diminished apoptin phosphorylation, whereas an AKT-specific inhibitor had no such effect. The activity of the AKT inhibitor was confirmed by Western blot analysis of AKT phosphorylation in the presence or absence of AKT inhibitor on total cell lysate from MM1.R cells (data not shown). The level of total apoptin detected by the apoptin antibody showed that the total amount of MBP-apoptin supplemented was similar in different samples. To obtain evidence that the PKCβ isoforms also phosphorylated apoptin in cells, we coexpressed apoptin with the catalytic domains of PKCβ1 and PKCβ2 (pPKCβ1-CF or pPKCβ2-CF) or control pBabePuro in 293T cells. At 48 hours after transfection, total cell extracts were separated on SDS-PAGE and protein expression was analyzed by Western blotting using anti-PKCβ1 or anti-PKCβ2 antibodies. The catalytic domain fragments (CF) of both PKCβ isoforms were detected by Western blotting with PKCβ variant–specific antibodies (Fig. 3D). In agreement with the *in vitro* data, overexpression of PKCβ-CF resulted in a significant increase in apoptin phosphorylation. In contrast, apoptin was weakly phosphorylated by cotransfection with the empty vector control, representing the basal phosphorylation levels in 293T cells. Anti-GFP antibody was used on the same blot to show similar expression levels of GFP-apoptin in different samples. Furthermore, cell death was increased when exogenous PKCβ1-CF or PKCβ2-CF were coexpressed with apoptin (Fig. 3D).

### shRNA-mediated knockdown or drug inhibition of PKCβ activity inhibits apoptin phosphorylation

To further show a causal link between PKCβ and apoptin phosphorylation, MM1.R cells were infected with LV-GFP-AP and lentiviral vectors expressing shRNA against PKCβ. The successful knockdown of endogenous PKCβ1 and PKCβ2 protein levels by shRNA was shown by Western blotting (Fig. 4A). A strong reduction in apoptin phosphorylation was observed at 3 days after transduction with shRNA. However, FACS analysis of PI-positive cells showed increased rather than decreased cell death in cells coexpressing apoptin together with PKCβ shRNA (Supplementary Fig. S3A and B). This is not surprising as PKCβ isoforms are known to play an important role in cell proliferation and their inhibition is likely to suppress cellular growth. Moreover, we examined PKCβ-mediated apoptin phosphorylation using PKCβ-specific or AKT-specific inhibitors (Fig. 4B). We found that apoptin phosphorylation was reduced 3 days after treatment with PKCβ inhibitor or AKT inhibitor. However, PKCβ inhibition

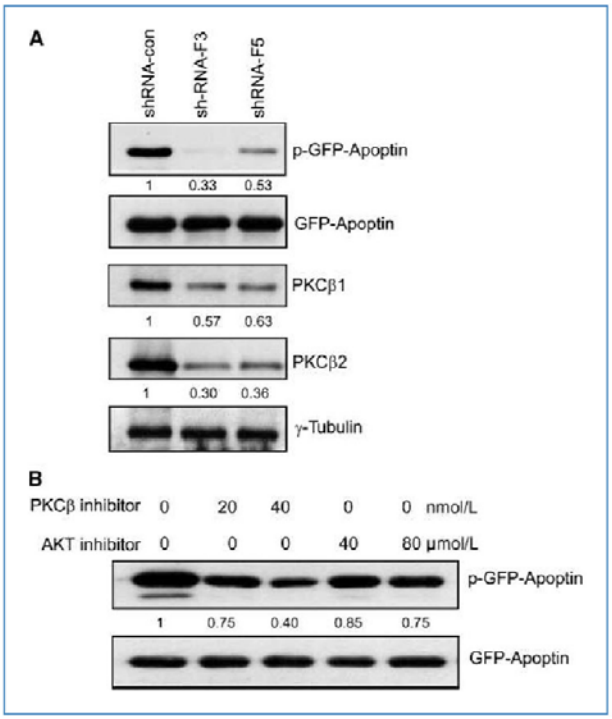


Figure 4. Apoptin phosphorylation is regulated by PKCβ expression and PKCβ activity. A, PKCβ knockdown resulted in reduced apoptin phosphorylation. MM1.R cells were coinfected with lentiviral vector encoding GFP-apoptin and lentiviral construct encoding either shRNA-con (no homology to human genome), or shRNA-F3 and shRNA-F5 directed against PKCβ. The levels of phosphorylated apoptin and the knockdown of PKCβ were detected by Western blotting of cell lysates obtained 3 d after infection. The same blot was probed to detect phosphorylated apoptin, GFP-apoptin, PKCβ1, PKCβ2, and γ-tubulin. B, apoptin phosphorylation is reduced by inhibition of PKCβ activity. MM1.R cells were infected with LV-GFP-AP. PKCβ and AKT inhibitors were applied on day 1 postinfection with the indicated concentrations; on day 3, the cell lysates were detected by immunoblotting with apoptin phosphospecific and GFP antibodies. Numbers below each lane represent the quantified level of protein as compared to the control.

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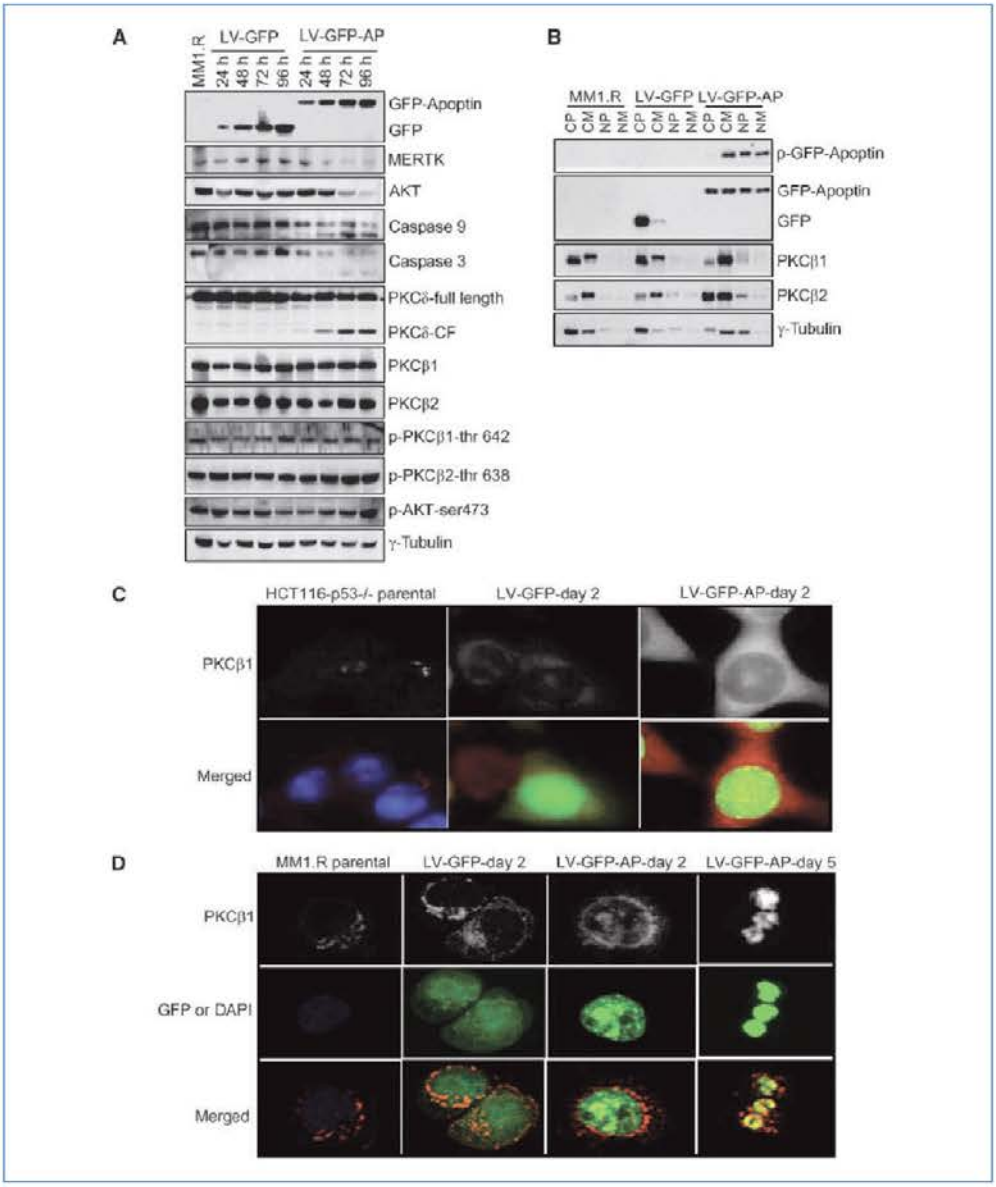


**Figure 5.** Apoptin differentially regulates cellular protein kinases. A, kinase protein levels and their activities detected by Western blotting from whole cell lysates of transduced MM1.R cells. B, protein levels detected by Western blotting from subcellular fractions of transduced MM1.R cells on day 3. CP, proteins isolated from the cytoplasmic fraction; CM, proteins from the cell membrane fraction; NP, proteins isolated from the nuclear fraction; NM, proteins bound to the nuclear membrane. C, apoptin upregulates PKCβ in HCT116-p53-/-. HCT116-p53-/- cells were fixed and labeled with anti-PKCβ1 antibody on day 2 postinfection. D, PKCβ colocalized with apoptin in the nucleus of transduced MM1.R cells. The cells were fixed and stained with anti-PKCβ1 antibody on the indicated days postinfection, and representative images were taken by fluorescence microscopy. Apoptin-expressing MM1.R cells clearly showed condensed apoptotic nuclei on day 5 postinfection.

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decreased phosphorylation of apoptin considerably more than inhibition of AKT. These results collectively suggest that PKCβ is an important apoptin kinase, but other intricate pathways may be involved in apoptin phosphorylation and regulation of its activation.

**Apoptin differentially regulates cellular kinases and kinase activities**

We further studied the effect of apoptin on the expression and activation of other cellular candidate kinases, which were either reported to play a role in the apoptin activity or were identified in our microarray expression profiling.

Infection of MM1.R cells with either control LV-GFP or LV-GFP-AP resulted in a time-dependent increase of GFP or GFP-AP expression. However, apoptin expression resulted in a decreased expression of MERTK and AKT as normalized to γ-tubulin (Fig. 5A). PKCβ1 and PKCβ2 protein levels remained unchanged, but increased phosphorylated PKCβ1, PKCβ2, and AKT were detected in total cell extracts. Meanwhile, elevated levels of PKCβ1 and β2 in cytoplasmic membrane and nuclear fractions, representing kinase activities, were also detected in the apoptin-transduced cells (Fig. 5B). Interestingly, apoptin expression also induced cleavage of caspase-9, caspase-3, and PKCδ, which correlated with apoptin-induced cell death.

Furthermore, we found that in tumor cells, the cytosolic fraction of the apoptin protein was not phosphorylated, whereas the plasma membrane-bound, nuclear, and nuclear membrane–associated apoptin protein was significantly phosphorylated. These data indicate that phosphorylation plays an important role in the subcellular localization of apoptin (see Fig. 5B). These membranes are the sites where PKCβ is considered to be active (not in the cytosol; ref. 30). Similar results were obtained with different cell lines infected with LV-GFP-AP (data not shown), suggesting that apoptin is phosphorylated by the activated PKCβ at cellular membranes.

It is widely accepted that the biological functions of PKC isoforms depend on their intracellular localization. PKC isoforms can be localized to multiple cellular compartments, including the plasma membrane, endosomes, endoplasmic reticulum, Golgi, nucleus, and nuclear membrane. To study the subcellular localization of PKCβ, MM1.R and p53-deficient HCT116 cells (sensitive to apoptin killing) were infected with LV-GFP (MOI 2) or LV-GFP-AP (MOI 8), respectively. The untransduced and GFP-transduced HCT116 cells showed a low level of cytosolic distribution of PKCβ, whereas the apoptin-transduced HCT116 cells had increased cytosolic levels and nuclear import of PKCβ (Fig. 5C). In the untransduced and GFP-transduced MM1.R cells, PKCβ mostly concentrated near the nucleus in a pattern that resembled the endoplasmic reticulum. In apoptin-transduced MM1.R cells, activated PKCβ relocated to the nuclear membrane and also translocated into the nucleus, colocalizing with apoptin. Apoptin-expressing MM1.R cells clearly showed condensed apoptotic nuclei on day 5 postinfection (Fig. 5D).

## Discussion

Recently, several viral and cellular proteins that specifically kill tumor cells have been identified. Such proteins as well as their cellular interacting and regulatory targets are important candidates for anticancer therapeutics. In the present study, we have identified a novel cellular pathway involved in the sensitization of cancer cells to apoptin.

Apoptin has been shown to be predominantly localized in the nucleus of cancer cells, whereas in normal cells its nuclear accumulation is severely impaired (13, 14). Several studies indicated that phosphorylation of apoptin is crucial for its nuclear localization and cytotoxic activity (9, 10). Here, we provide evidence that the phosphorylation and nuclear migration of apoptin in tumor cells is mediated by PKCβ. Deletion and point mutation studies have shown the importance of the COOH-terminal domain (amino acids 80–121) of apoptin for its nuclear accumulation and function (31–34). Apoptin was shown to be phosphorylated in this domain predominantly at Thr-108 in cancer cells (16, 17). Analysis of the apoptin amino acid sequence for potential phosphorylation sites using NetPhos software (http://www.cbs.dtu.dk/services/NetPhos) indicated seven putative phosphorylation sites corresponding to the PKC kinase consensus motifs (S/TXK/R or S/TXXK/R). These sites included Thr-108, which has been previously shown to be phosphorylated in tumor cells but not in normal cells (31, 32). Microarray expression profiling of multiple myeloma cell lines with different sensitivity to apoptin identified the PKCβ family as potential tumor-specific apoptin kinases. Our *in vitro* and *in vivo* kinase studies and the knockdown of PKCβ further confirmed that apoptin was phosphorylated by PKCβ.

Ludwig and colleagues have recently shown that virus infection or the treatment with the viral protein hemagglutinin resulted in a rapid activation of PKC isoforms (35). Furthermore, besides the well-established role of PKCs in activating the downstream RAF/MEK/ERK kinases, PKCs have been found to directly phosphorylate several viral proteins (36, 37). Our study provides further evidence that PKCβ may be an important cellular component that interacts with viral proteins including apoptin. Consequently, PKCβ phosphorylates apoptin and triggers its nuclear migration where it induces the activation of multiple signaling events, involving caspase-9, caspase-3 activation, and cleavages of PKCδ, shifting the equilibrium from survival signaling toward the activation of the cell death machinery.

Recently, apoptin has been shown to interact with the SH3 domain of p85 regulatory subunit of PI3K and its downstream effector AKT kinase (18, 19, 38). Interestingly, the interaction of apoptin with AKT seems to trigger the nuclear trafficking of AKT in parallel with apoptin, resulting in the AKT-mediated phosphorylation of downstream mitogenic cyclin-dependent kinase CDK2 and consequent phosphorylation of apoptin by CDK2 in the nucleus. However, in this study, we have been unable to show an interaction between apoptin and either the p85 subunit of PI3K or AKT, but we could not entirely rule out the involvement of PI3K/AKT pathway in apoptin phosphorylation either. Particularly, inhibition with an AKT inhibitor showed decreased apoptin

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phosphorylation to some extent. In our study, apoptin expression resulted in AKT activation and rapid AKT protein degradation, which may prevent detection due to a sensitivity problem. The inconsistencies between our study and that of Maddika and colleagues could be due to the different intrinsic cell features in the cell types used. MCF7 cells, which they used in their experiments, have previously been shown by others to be caspase-3 deficient (39). It has been reported that AKT kinases are functionally inactivated by caspase-3 cleavage in response to a variety of apoptotic stimuli and growth factor withdrawal (40, 41). A nuclear translocation of AKT with apoptin in MCF7 cells reported by Maddika and colleagues could therefore be due to the lack of cleavage of AKT in the caspase-3–deficient MCF7 cells, resulting in a full-length, active AKT that could capture and accompany apoptin into the nucleus.

In contrast to AKT, which was destabilized by apoptin, a consistent, stable PKCβ protein level was detected in MM1.R cells. It is generally accepted that the transient activation of PKCs supports cell survival, whereas a sustained activation of PKCs induces apoptosis (21). Activation of PKCs in certain leukemia cell lines (HL60, U937, and K562) by TPA has been shown to be associated with growth arrest and terminal differentiation (42–44). This effect is also often accompanied by apoptosis.

Controlled cellular proliferation involves multiple mechanisms that balance increased cell numbers with subsequent cell death. This complex process is orchestrated by many kinases and caspases (45). We observed increased cleavage of caspase-9, caspase-3, and PKCδ during apoptin-induced cell death in myeloma cell lines. Active caspase-8 or caspase-9 can directly cleave and activate effector caspases, such as caspase-3, and/or engage the intrinsic apoptotic pathway through the cleavage of the Bcl-2 homology 3 protein Bid (46). Cleaved Bid translocates to the mitochondria, where it triggers activation of the intrinsic apoptotic pathway by promoting activation of the Bcl-2 proteins Bax and Bak, which induces loss of mitochondrial membrane potential and release of proapoptotic mitochondrial components into the cytoplasm. PKCδ is a well-characterized and ubiquitously expressed kinase with multiple functions (47). Full-length PKCδ is located in the cytoplasm and has been shown to promote cell proliferation, whereas cleaved PKCδ is located in the nucleus and has proapoptotic functions. Exposure to numerous apoptotic stimuli results in the activation of PKCδ and its translocation to the nucleus. Our data indicate that apoptin activates both PKCδ and caspase-3 and enhances their nuclear accumulation (data not shown). In the nucleus, PKCδ seems to be cleaved by activated caspase-3 to generate the constitutively activated proapoptotic cleaved form (48). Potential target substrates of PKCδ are nuclear proteins that are involved in apoptotic cell death such as lamin B (nuclear structural protein), DNA-dependent protein kinase (DNA-PK), Rad9 (cell cycle checkpoint protein), p53, p73β, and STAT1 (transcription factors; refs. 20, 23, 47). Taken together, our data suggest that cleavage of PKCδ and caspase-3 is also involved in the regulation of apoptin-mediated apoptosis, but downstream of apoptin. This is consistent with previous reports showing that caspase-3 is required for apoptin-induced apoptosis (12).

Cancer remains one of the leading causes of death, and many cancer patients relapse as they become resistant to conventional therapies. There is mounting evidence that cancer cells, including leukemia, have an intrinsic ability to prevent apoptosis (49). Here, we provide important evidence that the ectopic expression of apoptin can restore the failing apoptosis program in leukemia and overcome intrinsic or acquired resistance to cell death. Furthermore, apoptin was able to effectively eliminate multiple myeloma cells that had become resistant to dexamethasone. This study has led to the identification of tumor-specific cellular targets such as PKCβ, whose modulation by shRNAs and small-molecule drugs can induce strong antileukemia effects.

In conclusion, the present study provides novel mechanisms for apoptin regulation by protein phosphorylation involving the PKC pathway. This knowledge can be applied to understanding the role of these kinases in response to treatment with a variety of anticancer agents. Importantly, the evidence that our newly developed lentiviral vector expressing apoptin has no effect on $CD34^+$ progenitor colony formation while effectively killing multiple myeloma cells further supports apoptin as an important and ideal *ex vivo* purging agent in the autologous bone marrow transplantation for human multiple myeloma.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Human Gyrovirus Apoptin shows a similar subcellular distribution pattern and apoptosis induction as the chicken anaemia virus derived VP3/Apoptin

J Bullenkamp[1,3], D Cole[1,3], F Malik[2], H Alkhatabi[2], A Kulasekararaj[2], EW Odell[1], F Farzaneh[2], J Gäken[2,3] and M Tavassoli*[,1,3]

**The chicken anaemia virus-derived protein Apoptin/VP3 (CAV-Apoptin) has the important ability to induce tumour-selective apoptosis in a variety of human cancer cells. Recently the first human Gyrovirus (HGyV) was isolated from a human skin swab. It shows significant structural and organisational resemblance to CAV and encodes a homologue of CAV-Apoptin/VP3. Using overlapping primers we constructed a synthetic human Gyrovirus Apoptin (HGyV-Apoptin) fused to green fluorescent protein in order to compare its apoptotic function in various human cancer cell lines to CAV-Apoptin. HGyV-Apoptin displayed a similar subcellular expression pattern as observed for CAV-Apoptin, marked by translocation to the nucleus of cancer cells, although it is predominantly located in the cytosol of normal human cells. Furthermore, expression of either HGyV-Apoptin or CAV-Apoptin in several cancer cell lines triggered apoptosis at comparable levels. These findings indicate a potential anti-cancer role for HGyV-Apoptin.**

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Apoptin was originally identified as the death-inducing protein VP3 from chicken anaemia virus (CAV), the first member of the Gyrovirus genus. Infection with the virus, which is mainly transmitted through contaminated feathers, has been shown to trigger apoptosis in thymocytes and myeloid progenitor cells of young chicken.[1] CAV is a single-stranded DNA virus which encodes three proteins, VP1 (capsid protein), VP2 (protein phosphatase and scaffold protein) and VP3.[2] *In vitro*, expression of VP3 alone induces apoptosis of chicken lymphoblastoid T-cells and myeloid cells in a similar way as observed after CAV infection.[3,4] Hence, VP3 was renamed Apoptin.

CAV-Apoptin is a 121 amino-acid protein rich in proline, serine, threonine and basic amino acids.[5,6] It contains a bipartite-type nuclear localisation signal (NLS1 and NLS2) at position 82-88 and 111-121 and a nuclear export signal (NES) suggesting potential shuttling of the protein between nucleus and cytoplasm.[7,8] Both predicted NLSs of Apoptin are required for its efficient nuclear localisation.[7] Furthermore, Apoptin contains several potential phosphorylation sites, including threonine 108.[9]

Importantly, Apoptin has the ability to induce apoptosis in various human cancer cells but not in normal cells.[10–12] It triggers apoptosis through the intrinsic mitochondrial pathway requiring caspase-3 and caspase-9, but not caspase-8. Cell death is induced independently of p53 but possibly involves the p53 family member p73 that can transactivate pro-apoptotic targets such as Bax and Puma.[13,14] Furthermore, the sphingomyelin-ceramide pathway has been shown to have a role in Apoptin-induced cytotoxicity.[15] However, the precise cellular mechanisms of Apoptin-induced cell death and the mode of tumour selectivity remain unclear. There are distinct differences regarding Apoptin expression between normal and tumour cells, which may provide clues about the mechanisms of tumour cell sensitivity to Apoptin. Firstly, in tumour cells Apoptin localises to the nucleus, which is important for its ability to induce apoptosis, whereas in normal cells it is predominantly expressed in the cytoplasm.[10,16] In addition, Apoptin is phosphorylated on threonine 108 in tumour but not in normal cells, which drives nuclear accumulation in tumour cells.[9] The specific kinase/s responsible for Apoptin phosphorylation remain unidentified. However, several kinases, including PKC$\beta$ and CDK2 have been shown to phosphorylate Apoptin.[17,18]

Recently, a virus showing significant homology to CAV was isolated from skin swabs of healthy volunteers and designated human Gyrovirus or HGyV.[19] Similar to CAV the HGyV genome consists of a single-stranded negative-sense circular DNA of 2.315 nucleotides (compared with 2.290–2.320 for

[1]Head and Neck Oncology Group, Department of Oral Pathology, King's College London Dental Institute, Guy's Hospital Campus, London SE1 9RT, UK and [2]Department of Haematological Medicine, The Rayne Institute, King's College London,123 Coldharbour Lane, London SE5 9NU, UK
*Corresponding author: M Tavassoli, Head and Neck Oncology Group, Department of Oral Pathology, Dental Institute, King's College London, Guy's Hospital, London SE1 9RT, UK. Tel: +44 (0)271888321; Fax: +44 (0)271884375; E-mail: mahvash.tavassoli@kcl.ac.uk
[3]These authors contributed equally to this work.
**Keywords:** apoptin; human gyrovirus; apoptosis; tumour specificity; cancer therapy
**Abbreviations:** CAV, chicken anaemia virus; HGyV, human Gyrovirus; GFP, green fluorescent protein; NLS, nuclear localisation signal; NES, nuclear export signal; LRS, leucine rich stretch; PKC$\beta$, protein kinase c beta; CDK2, cyclin dependent kinase 2; HGyV-AP, HGyV-derived Apoptin; GFP-AP, GFP-tagged Apoptin; FLAG-AP, FLAG-tagged Apoptin; PI, propidium iodide; APC, allophycocyanin; 1BR3LT, SV40 large T transformed 1BR3 fibroblasts; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; CIP, calf intestinal phosphatase
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CAV), containing three partially overlapping open reading frames. Alignment with CAV revealed relatively low overall sequence identity with a maximal identity of 70% in the region of nucleotides 100–700 but a similar organisation of the promoter region and the encoded proteins. In addition to VP1 and VP2 HGyV encodes a slightly larger, 125 amino-acid homologue of CAV-Apoptin/VP3 (121 amino acids for comparison). Despite a low overall identity important regions such as the nuclear localisation and export signals and phosphorylation sites are conserved between HGyV- and CAV-Apoptin. The effects or involvement of this novel virus in human disease and the functional properties of human Gyrovirus Apoptin (HGyV-Apoptin) are currently unknown.

To investigate whether HGyV-Apoptin has apoptotic and tumour selective activity similar to its homologue CAV-Apoptin we developed a synthetic HGyV-Apoptin fused to green fluorescent protein (GFP). Expression of this construct in human cancer cell lines revealed a subcellular localisation and pro-apoptotic function comparable to CAV-Apoptin.

## Results

**Expression of HGyV-Apoptin in human cancer cells.** To test the expression of the newly generated HGyV-Apoptin constructs, HCT116 colon carcinoma and Saos-2 osteosarcoma cells were transfected with the corresponding plasmids pHGyV-GFP-AP and pHGyV-FLAG-AP. Western blot analysis of transfected cells after 2 days revealed expression of both HGyV-GFP-AP and HGyV-FLAG-AP that could be detected at the estimated molecular weights (Figures 1a and b). Compared with its CAV homologue HGyV-GFP-AP is slightly larger as expected from the difference in length. Re-probing with a CAV-Apoptin phospho-specific antibody directed against phosphorylated threonine 108 did not reveal any cross-reaction with HGyV-Apoptin (data not shown).

**HGyV-Apoptin translocates to the nucleus and induces changes in nuclear morphology in cancer cells.** CAV-Apoptin is known to translocate to the nucleus in transformed cells which is at least partially important for the induction of apoptosis in these cells. To investigate the subcellular distribution of HGyV-Apoptin, which also contains putative nuclear localisation and export signals (Figure 1a), as well as morphological changes caused by its expression, cells were transfected with different Apoptin constructs and analysed by fluorescence microscopy. Imaging of HCT116 colon carcinoma cells transfected with pCAV-GFP-AP or pHGyV-GFP-AP at 24 and 48 h post transfection revealed a similar

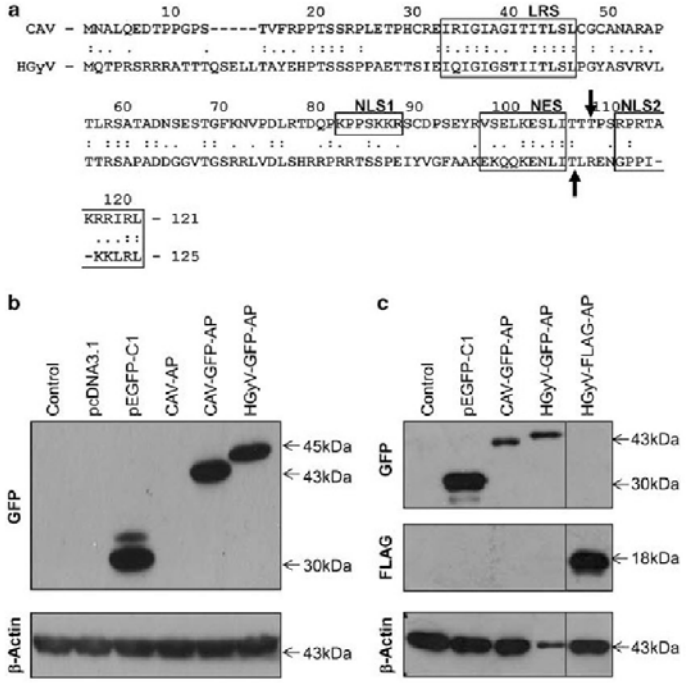


**Figure 1** Expression of CAV- and HGyV-Apoptin in cancer cells. (**a**) Alignment of the protein sequence of CAV-Apoptin (NP_056774.1) and HGyV-Apoptin (CBZ41794.1) using computer software Align (http://xylian.igh.cnrs.fr/bin/align-guess.cgi). Important functional domains, including LRS (leucine-rich domain), NLS1/2 and NES are indicated by boxes and the predicted phosphorylation sites threonine 108 or threonine 111 are indicated by black arrows. Whole cell lysates of HCT116 colon carcinoma (**b**) and Saos-2 osteosarcoma (**c**) cells transfected with the indicated plasmids were prepared and western blot analysis for detection of GFP- or FLAG-Apoptin, respectively, as well as *β*-Actin as a loading control was performed. Molecular weights of the bands are indicated with arrows and blots were cut and combined at the black line

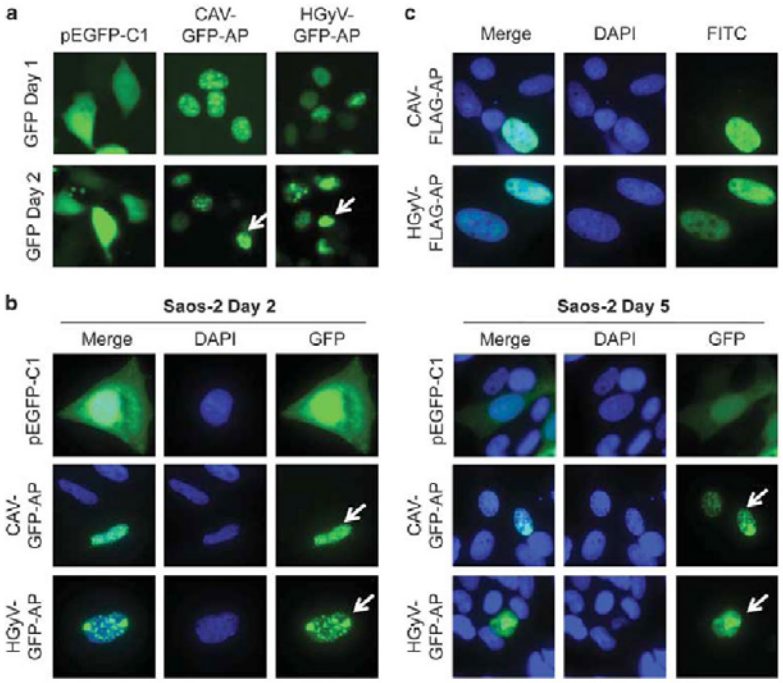


**Figure 2** Nuclear translocation of GFP- and FLAG-Apoptin in cancer cells. HCT116 (**a**) and Saos-2 (**b**) cancer cells were transfected with pCAV-GFP-AP or pHGyV-GFP-AP and the corresponding pEGFP-C1 control plasmid. Cells were directly imaged in culture dishes after 1 or 2 days (**a**) or fixed and counterstained with DAPI at 2 and 5 days post-transfection (**b**) as indicated. Saos-2 cells (**c**) transfected with pCAV-FLAG-AP or pHGyV-FLAG-AP were fixed after 5 days and stained with a primary mouse anti-FLAG and secondary FITC anti-mouse antibody for detection of FLAG-Apoptin. Nuclei were detected by counterstaining with DAPI. Arrows indicate apoptotic nuclei

expression pattern of CAV- and HGyV-Apoptin (Figure 2a). CAV-Apoptin has been shown to initially exhibit a fine granular distribution within the nucleus, later clustering to form aggregates, at which point the cell becomes apoptotic. This characteristic morphology was also observed with HGyV-Apoptin in transfected Saos-2 osteosarcoma cells after 2 and 5 days, respectively (Figure 2b). To exclude a potential promoting effect of the GFP-tag on nuclear translocation Saos-2 cells were transfected with FLAG-CAV-AP or FLAG-HGyV-AP. Subsequent indirect immunofluorescence to detect FLAG-Apoptin revealed the same expression pattern as observed for GFP-Apoptin (Figure 2c). Similar cellular distribution was observed in the HT1080 breast cancer cell line (data not shown).

**HGyV-GFP-Apoptin induces cell death in Saos-2 osteosarcoma cells.** To examine the cytotoxic function of HGyV-GFP-AP Saos-2 human osteosarcoma cells were transfected by nucleofection with pHGyV-GFP-AP, pCAV-GFP-AP or the corresponding pEGFP-C1 control vector. Apoptosis was quantified by flow cytometric analysis as percentage of Annexin-V-positive cells among the transfected cells after Annexin-V-APC/ propidium iodide (PI) staining. After 5 days between 40 and 50% of cells expressing GFP-Apoptin were apoptotic compared with only 5% apoptotic cells in those transfected with pEGFP-C1 (Figure 3a). The level of cell death induced by HGyV-Apoptin was similar to the level achieved by CAV-Apoptin.

Furthermore, a colony-forming assay of HT1080 cells comparably transfected with pEGFP-C1 or pHGyV-GFP-AP showed very few HGyV-GFP-AP expressing cells, whereas the control pEGFP-C1 transfected cells were able to form countless colonies in the presence of G418 (data not shown) indicating a cytotoxic effect of HGyV-GFP-AP.

To compare the pro-apoptotic effect of GFP- and FLAG-tagged Apoptin, Saos-2 cells were transfected with different Apoptin and control plasmids. After 5 days cells were fixed, stained with anti-FLAG antibody (for pCAV-FLAG-AP and pHGyV-FLAG-AP) and counterstained with DAPI for the detection of nuclear morphology. Apoptosis was quantified by scoring cells expressing either GFP- or FLAG-Apoptin and displaying clear apoptotic nuclear morphology. Results suggested that both CAV- and HGyV-Apoptin efficiently induce apoptosis as compared with the pEGFP-C1 control. Furthermore, no significant differences between the GFP- or FLAG-tagged Apoptin could be observed (Figure 3c).

**HGyV-GFP-Apoptin is expressed in the cytoplasm of normal human fibroblasts and translocates to the nucleus of transformed counterparts.** One of the

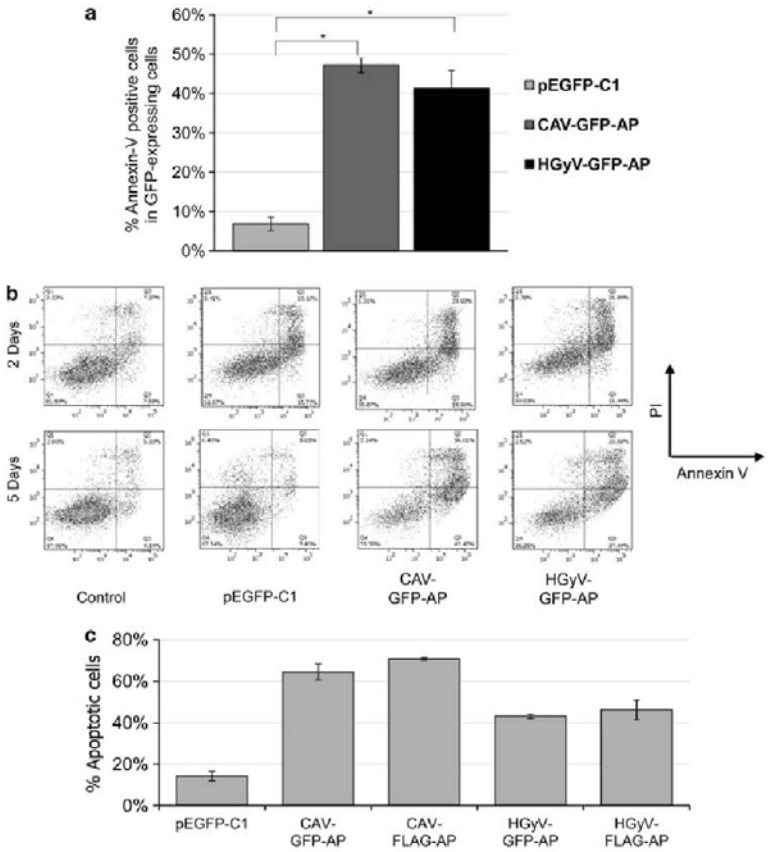


**Figure 3** Induction of cell death in Saos-2 cells at 5 days after transfection. Saos-2 cells were transfected by nucleofection with pCAV-GFP-AP, pHGyV-GFP-AP or the corresponding pEGFP-C1 control plasmid, as well as pCAV-FLAG-AP and pHGyV-FLAG-AP. Cell death was measured by flow cytometric analysis after Annexin-V-APC/ PI staining. (**a**) Quantification of cell death as % of APC-labelled cells in the GFP-positive population after 5 days (*$P < 0.01$). Error bars indicate standard deviation of three experiments. (**b**) Corresponding FACS dot plots of Annexin-V-APC (x-axis) *versus* PI (y-axis) obtained after 2 and 5 days. (**c**) After 5 days transfected Saos-2 cells were fixed, stained with a primary mouse anti-FLAG and secondary FITC anti-mouse antibody (for pCAV-FLAG-AP and pHGyV-FLAG-AP) and counterstained with DAPI for the detection of nuclear morphology. Cell death was quantified as the percentage of GFP- or FLAG-positive cells showing condensed or fragmented nuclei. Error bars indicate standard deviation of two independent experiments

important characteristics of CAV-Apoptin is its distinct tumour specific nuclear localisation, which is necessary for its ability to selectively induce apoptosis in transformed but not in normal cells. To investigate whether HGyV-Apoptin shows the same tumour selectivity pEGFP-C1, pHGyV-GFP-AP, pCAV-GFP-AP, pHGyV-FLAG-AP and pCAV-FLAG-AP were transfected into 1BR3 normal human fibroblasts and their SV40-LT transformed counterparts, (1BR3LT). After 3 days cells were counterstained with DAPI and analysed by fluorescent microscopy to quantify apoptosis by scoring the GFP-positive cells containing condensed or fragmented nuclei. The normal 1BR3 fibroblasts exhibited a marked difference in cellular localisation of both CAV-GFP-AP and HGyV-GFP-AP as compared with transformed 1BR3LT (Figure 4a). In normal cells, HGyV- and CAV-GFP-Apoptin were mainly expressed in the cytoplasm, while in the transformed cells both CAV- and HGyV-GFP-Apoptin had a predominantly nuclear localisation. Similarly, FLAG-tagged CAV- or HGyV-Apoptin was localised to the nucleus of transformed 1BR3LT cells, whereas it was predominantly cytoplasmic in normal 1BR3 fibroblasts (data not shown). This difference in subcellular localisation was associated with increased apoptosis in the transformed cells transduced with either FLAG- or GFP-Apoptin (Figure 4b).

## Discussion

Tumour specificity remains one of the main hurdles for successful treatment of cancer.[20] A number of viral and

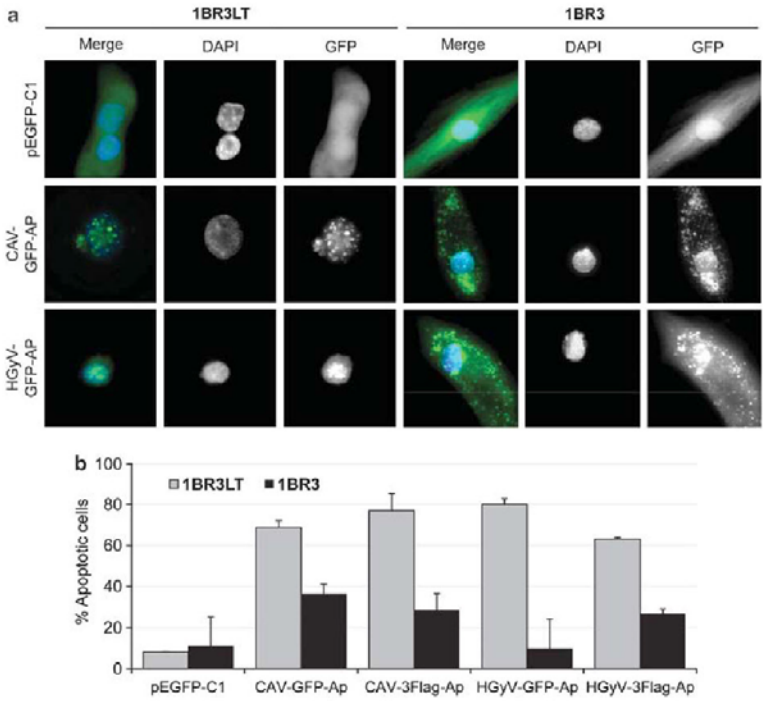


**Figure 4** Cytosolic expression of GFP-Apoptin and quantification of apoptosis in 1BR3 normal human fibroblasts. Normal (1BR3) and transformed (1BR3LT) human fibroblast cell lines were transfected by nucleofection with pCAV-GFP-AP, pHGyV-GFP-AP or the corresponding pEGFP-C1 control plasmid as well as pCAV-FLAG-AP and pHGyV-FLAG-AP. After 3 days cells were fixed and counterstained with DAPI to analyse the subcellular localisation of GFP-Apoptin. Expression of FLAG-AP was detected using primary mouse anti-FLAG and secondary FITC anti-mouse antibody (**a**). Apoptosis was quantified by scoring GFP- or FLAG-positive cells containing condensed or fragmented nuclei (**b**). Over one hundred transfected cells were analysed and experiments were repeated twice (error bars indicate standard deviation)

cellular proteins have been described that show tumour-specific toxicity, including Apoptin, Trail, mda-7 (etc).[21] The therapeutic potential of such proteins and their cellular targets is of immense interest. Recently, a HGyV has been identified encoding a viral protein resembling the CAV-derived Apoptin. Here we provide clear evidence that this HGyV-Apoptin shows a tumour-specific nuclear expression pattern as has been described previously for CAV-Apoptin to be important for its cytotoxicity.[7] Furthermore, expression of HGyV-Apoptin resulted in the induction of apoptosis in transformed cells but not in the normal human cell line 1BR3. However, further studies, including the use of different types of normal cells, are necessary to confirm that HGyV-Apoptin has tumour-selective cytotoxicity and could thus provide a novel tool for cancer treatment.

Many studies in the past decade have provided insight into the mechanism of Apoptin-induced apoptosis. However, the basis for its increased sensitivity for cancer cells remains largely unclear. In addition to translocation to the nucleus, CAV-Apoptin is phosphorylated on threonine 108 in cancer cells but not in normal cells, which seems to be important for enabling Apoptin to induce apoptosis.[9] Several cellular kinases have been proposed to be responsible for CAV-Apoptin phosphorylation.[14,18] We have recently identified PKC$\beta$ to be important for phosphorylation and activation of CAV-Apoptin.[17] It would be important to investigate whether HGyV-Apoptin is phosphorylated in a similar manner by the kinases which have been shown to interact with CAV-Apoptin. These studies are currently on-going.

Although so far no link between this novel HGyV and any human disease has been identified, the clear apoptotic activity of its encoded protein HGyV-Apoptin offers an intriguing opportunity to investigate its potential pathological role. Furthermore a better understanding of the function of the viral protein HGyV-Apoptin may reveal involvement in human disease and lead to the development of novel therapeutics for cancer.

## Materials and Methods

**Cell lines and cell culture.** HCT116 human colorectal carcinoma cells were obtained from ATCC (Manassas, VA, USA) and maintained in McCoy's 5A medium supplemented with 10% foetal bovine serum 40 U/ml Penicillin and 40 $\mu$g/ml Streptomycin (all PAA Laboratories, Yeovil, UK). Saos-2 human osteosarcoma cells were purchased from ATCC and grown in DMEM (PAA Laboratories) supplemented with 10% foetal bovine serum 40 U/ml Penicillin and 40 $\mu$g/ml Streptomycin. 1BR3 normal and 1BR3 SV40 1BR3LT were a gift from Prof. Alan Lehmann (Sussex Centre for Genome Damage and Stability, University of Sussex, UK) and maintained

**Table 1** Forward and reverse primers for the generation of HGyV-Apoptin

| *Primer* | *Sequence* |
|---|---|
| FOR1 | 5′-GGATCCGCCGCCACCATGCAGACCCCCSGC TCTCGCAGAAGAGCCACCACCACCCAGTCC-3′ |
| FOR2 | 5′-AGCTCCTCTCCCCCAGCCGAAACCACCTCC ATCGAGATCCAGATTGGAATCGGGTCCACT-3′ |
| FOR3 | 5′-AGCGTGCGCGTGCTCACCACCAGATCTGCA CCTGCCGACGATGGAGGAGTCACTGGGTCC-3′ |
| FOR4 | 5′-CCCRGACGGACCAGCTCTCCAGAGATCTAC GTCGGCTTCGCCGCAAAGGAGAAGCAGCAG-3′ |
| FOR5 | 5′-GGACCCCCTATCAAGAAGCT GAGACTGTAAATTTGAATTC-3′ |
| REV1 | 5′-TGGGGGAGAGGAGCTTGTTGGGTGCTCGTA GGCTGTCAGCAGCTCGGACTGGGTGGTGGT-3′ |
| REV2 | 5′-GAGCACGCGCACGCTGGCGTAGCCAGGCA GGGACAGAGTAATGATAGTGGACCCGATTCC-3′ |
| REV3 | 5′-GCTGGTCCGTCYGGGTCTCCTGTGTGACAG GTCCACCAGCCTCCTGGACCCAGTGACTCC-3′ |
| REV4 | 5′-CTTGATAGGGGGTCCGTTTTCCCTCAGGG TAATGAGGTTTTCTTTCTGCTGCTTCTCCTT-3′ |
| REV5 | 5′-GAATTCAAATTTACAGTCTCAGCTT-3′ |

in DMEM containing 15% foetal bovine serum 40 U/ml Penicillin and 40 $\mu$g/ml Streptomycin. All cell lines were incubated at 37 °C and 5% $CO_2$.

**Generation of HGyV-Apoptin.** A codon optimised HGyV-Apoptin gene was constructed using primers overlapping by 15 bases (Table 1). FOR1 introduces a Kozak sequence upstream of the ATG start codon and a *Bam*HI site for subsequent cloning. REV5 introduces an *Eco*RI site for subsequent cloning. Primers FOR1-5 and REV1-4 were mixed in equimolar amounts and heated to 95 °C for 2 min and cooled slowly to room temperature. Gaps were filled in with T4 DNA Polymerase for 15 min at 12 °C and finally the gaps were closed by treatment with T4 DNA Ligase for 15 min at RT. The resulting product was PCR amplified with primers FOR1 and REV5 and the PCR product was TOPO TA cloned (Invitrogen Life Technologies, Paisley, UK).

Inserts were sequence verified and a correct HGyV-AP was removed from the TOPO vector by *Bam*HI/*Eco*RI digestion and cloned in-frame with GFP into the *Bgl*II/*Eco*RI digested mammalian expression vector pEGFP-C1 (Clontech, Saint-Germain-en-Laye, France) and named pHGyV-GFP-AP.

pHGyV-FLAG-AP was cloned by removing HGyV-AP from the TOPO vector by *Eco*RI digestion and filling in with Klenow. p3X-FLAG (Sigma-Aldrich, Poole, UK) was digested with *Bam*HI and *Eco*RI, blunt ended with Mungbean nuclease and phosphates were removed by calf intestinal phosphatase treatment. Vector and insert were blunt end ligated and the correct orientation was determined by *Bst*XI and *Bam*HI digestion. Plasmids containing an insert in the correct orientation were sequence verified.

**Plasmids and transient transfection.** Expression vectors for HGyV-Apoptin (pHGyV-GFP-AP, pHGyV-FLAG-AP) were constructed as part of this study. pCAV-GFP-AP was generated as described previously[10] and pCAV-FLAG-AP was received from Dr. Jose Teodoro (Goodman Cancer Research Centre, Montreal, QC, Canada). Other control vectors used were pcDNA3.1 (Invitrogen) and pEGFP-C1.

HCT116 cells were transiently transfected using X-tremeGENE HP transfection reagent according to the manufacturer's protocol (Roche Diagnostics, Burgess Hill, UK). Transfection of Saos-2 and 1BR3/1BR3LT cells was achieved using Nucleofector technology as recommended by the manufacturer (Lonza, Basel, Switzerland).

**Flow cytometric analysis.** Cells were seeded on 12-well plates and collected at 2 or 5 days after nucleofection. Apoptosis was quantified by staining with Annexin-V-APC (Invitrogen) and PI (Sigma) and subsequent analysis on a BD FACSCanto II (Becton Dickinson, Oxford, UK). Data was analysed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

**Western blot analysis.** Protein extracts were prepared by washing cells and directly lysing them in Laemmli sample buffer (62.5 mM Tris-HCl pH 6.7, 100 mm $\beta$-mercaptoethanol, 2% SDS, 1 $\mu$g/ml aprotinin, 1 $\mu$g/ml leupeptin, 100 $\mu$g/ml phenylmethylsulphoxide). SDS-PAGE was performed and proteins were transferred to nitrocellulose membranes (Sigma). Membranes were incubated with specific primary and secondary antibodies as indicated and developed using a home-made enhanced chemiluminescent system. The antibodies used were: rabbit anti-GFP (Cell Signaling, Beverly, MA, USA), mouse anti-FLAG (Sigma), mouse anti-$\beta$-Actin (Sigma) and secondary anti-mouse (Sigma) or anti-rabbit (GE Healthcare, Chalfont St. Giles, UK) linked to horseradish peroxidase.

**Fluorescence microscopy.** HCT116 cells were imaged directly on culture plates at 1 or 2 days post-transfection using an Olympus CKX41 inverted microscope (Southend-on-Sea, UK).

Saos-2 and 1BR3/1BR3LT cells were transfected by nucleofection and grown on 8-well culture slides (Becton Dickinson). After 3 or 5 days cells were washed once with PBS, fixed in 2% paraformaldehyde for 30 min at 37 °C and permeabilized with 0.2% Triton X-100 for 20 min. For the detection of FLAG tag cells were washed in PBS, blocked using 3% BSA in PBS-T for 30 min at 37 °C and incubated with the primary anti-FLAG antibody and the secondary antibody anti-mouse-FITC (both Sigma) for 2 h or 1 h, respectively, at 37 °C with washing steps in PBS between the incubations. All cells were mounted in Vectashield mounting medium containing DAPI (Vector Laboratories, Peterborough, UK) for nuclear staining and images were acquired using an Olympus BX61 automated fluorescence microscope.

Apoptosis was quantified by scoring the GFP-positive cells containing condensed or fragmented nuclei. Over one hundred transfected cells were analysed and experiments were repeated twice.

**Statistical analysis.** A paired two-tailed Student's *t*-test was used to calculate significance.

## Conflict of Interest

The authors declare no conflict of interest.

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